TNF-α Signaling Regulates the Transcription of E-box Driven Clock Genes

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von

Saskia Petrzilka

von

Winterthur ZH

Promotionskomitee

Prof. Dr. Adriano Fontana (Vorsitz und Leitung der Dissertation)

Prof. Dr. Urs Greber

Prof. Dr. Roger Nitsch

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1. ZUSAMMENFASSUNG

Die biologische zirkadiane Uhr ist ein hochorganisiertes Zeitmessungssystem, welches zwischen allen Organismen konserviert ist. Die Tag- und Nachtzeit ist in jeder einzelnen Zelle genetisch festgehalten. Negative und positive Rückkoppelungsschlaufen der Genexpression und verschiedene post-translationelle Modifikationen der Uhrenproteine sind für einen ungefähren 24 Stunden Rhythmus verantwortlich.

Das zirkadiane System hilft Individuen ihr Verhalten an die täglichen Umweltveränderungen anzupassen und physiologische wie auch metabolische Bedürfnisse auf spezifische Zeitfenster abzustimmen. Allerdings müssen diese Prozesse nicht nur auf die äusseren Bedingungen abgestimmt werden, sondern sollten flexibel bleiben und die Fähigkeit besitzen auf neue Ansprüche des Körpers, im Falle eines Angriffs durch Mikroben, zu antworten.

Wie die Anpassung an die geophysikalische Zeit erfüllt wird, wurde schon gut untersucht. Lichtimpulse, welche auf die Retina treffen, werden über den retinohypothalamischen Gang an den suprachiasmatischen Nukleus (SCN) weitergeleitet. Durch intrazelluläre Signalwege, wie die Aktivierung von Kinasen und die Freigabe von Kalzium, wird der Uhren-Genexpressionszyklus gestartet. Daraufhin leitet der SCN seine Zeitinformation an die Peripherie weiter. Wie hingegen zirkadiane Uhren auf eine Immunantwort gegen Mikroben antworten, wurde bis jetzt nicht untersucht. Hinweise auf einen Zusammenhang zwischen dem Immunsystem und dem zirkadianen System ist daraus ersichtlich, dass sowohl die Produktion des pro-entzündlichen Zytokin Tumor Nekrose Faktor (TNF)-α bei einer Infektion als auch eine Dysregulation des zirkadianen Systems zu ähnlichen Symptomen führen wie z.B. Depressionen, Müdigkeit und Schlafstörungen.

Das Ziel meiner Doktorarbeit war es, den Effekt von TNF-α auf die molekulare zirkadiane Uhr zu untersuchen und die zu Grunde liegenden intrazellulären Mechanismen zu erforschen. Dazu wurden Fibroblastenkulturen mit TNF-α stimuliert und die Expression der Uhrengene analysiert. Diese Gene waren stark inhibiert, jedoch ohne dass dessen Periodizität verändert wurde. Interessanterweise waren nur

E-box abhängige Gene betroffen. Der Einfluss dieser Kontroll-Elemente in dem TNF-α induzierten Inhibitionsmechanismus konnte zusätzlich mit Luziferase Reporter Versuchen bestätigt werden. Dabei wurden wildtyp und mutierte E-boxen verglichen. Im Bestreben die exakte Signalkaskade, welche zu den beobachteten Änderungen führt, zu definieren, wurden in den darauffolgenden Experimenten verschiedene Inhibitoren gegen intrazelluläre Signalkomponenten und Zellen mit spezifischen Gendefekten eingesetzt. Es konnte eine Rolle der zentralen negativen Uhrenregulatoren im E-box abhängigen Inhibitionsmechanismus ausgeschlossen werden. Der TNF-α Rezeptor 1 und intrazelluläres Kalzium stellten sich als wichtige Elemente in der Uhrengen Regulation heraus. Hingegen konnte ein Beitrag der wichtigsten TNF-α Signalwege NF-κB, AP-1, oder Kaspase, zumindest in der frühen Antwort, ausgeschlossen werden. Diese Resultate weisen darauf hin, dass Kalzium nicht nur in der SCN-Uhr, wo es umweltbedingte Zeitsignale übermittelt, eine Schlüsselrolle besitzt; sondern möglicherweise auch eine zentrale Komponente in der peripheren Uhr ist, wo es immunologische Veränderungen wahrnimmt.

Es ist wahrscheinlich, dass der Körper im Falle einer Immunreaktion die physiologischen Prozesse zeitlich den neuen Bedingungen anpasst. Durch das Aufklären des genauen Mechanismus, mit welchem TNF-α die Uhr unterdrückt, wird es möglich sein in die Kaskade einzugreifen und somit spezifische Nebenwirkungen bei Patienten mit chronisch entzündlichen Krankheiten zu lindern.

2. SUMMARY

The circadian clock is a highly organized timekeeping system that is well conserved across all organisms. The time of the day is marked genetically in every individual cell. Interlocked negative and positive gene expression feedback loops and several posttranslational modifications of clock proteins are responsible for an almost 24 hour rhythm.

The circadian system helps individuals to adapt their behavior to the daily environmental changes and adjusts physiological and metabolic needs to specific time windows. Sometimes, however, these processes not only have to be adapted to the environment but also need to be flexible and able to respond to new exigencies of the body when it is attacked by microbes.

How the adaptation to the geophysical time is achieved has been the subject of many investigations already. Light inputs to the retina are transmitted to the suprachiasmatic nuclei (SCN) via the retinohypothalamic tract. By intracellular signaling, comprising kinase activation and calcium release, the clock gene expression cycle is launched. The SCN, in turn, further conveys its time information to the periphery. How circadian clocks are responding to an immune answer to microbes has not been investigated so far. A clear indication for cohesion between the immune and the circadian system is apparent because the production of the pro-inflammatory cytokine tumor necrosis factor (TNF)-α upon infection and disruption of the circadian system both lead to similar symptoms such as depression, fatigue and sleep disturbances.

The goal of my thesis was to investigate the effect of TNF-α on the molecular circadian clock and to further explore the intracellular mechanisms involved. Therefore, TNF-α was administered to a fibroblast cell culture and clock gene expression was found to be strongly dampened without affecting the periodicity. Interestingly, only E-box dependent genes were affected. The implication of these enhancer elements in the down regulation mechanism could be further confirmed by luciferase reporter assays comparing wild type to mutated E-boxes. In following experiments various blockers of intracellular signaling components and specific gene knock-out cells were used in the attempt to elucidate the precise signaling cascade

leading to the observed alterations. Thereby, a role for the central negative clock regulators in the E-box dependent inhibition mechanism could be excluded. The implication of the TNF-α receptor 1 and intracellular calcium were shown to be crucial for clock gene regulation by this cytokine. On the contrary, a contribution of the main TNF-α pathways NF-κB, AP-1 or caspase were clearly excluded at least in the early response. These results indicate that calcium is not only a key component of the SCN clock where it conveys environmental time signals, but possibly also plays a central role in the peripheral clock where it senses immunological changes.

It is likely that the body timely readapts physiological processes in the case of an immune reaction. By elucidating the exact mechanism through which TNF-α dampens the clock it will be possible to intervene in the cascade to alleviate specific side symptoms of patients with chronic inflammatory diseases.

3. INTRODUCTION

3.1. CIRCADIAN RHYTHMS

3.1.1. Why having a circadian clock?

All organisms studied so far have a system to tell the time of the day. This exists either in mammals, unicellular eukaryotes or prokaryotes, plants or fungi, even organisms that live deeply in caves and never see the sunlight. It is based on a circadian system: "circadian" derives from the Latin words *circa* and *diem* which together mean "about a day". It is vitally important to be able to anticipate the coming of a certain time of a day. For example, bats living in a cave, which is not reached by sunlight, need to have a clock system to know when it is time to come out for eating and preys should hide before it is time for the predators to chase. Bees have to know which plants flower at what time and plants have to prepare their photo-system before sunrise so that they can start photosynthesis with the first sunbeam. These are all good reasons for organisms depending on the solar system, but what about the organisms that never are in contact with light? The idea is that a circadian system optimizes cellular physiology by distributing biochemical incompatible reactions to different times of the day, or why should glycogen synthesis and degradation happen at the same time?

3.1.2. History of clock

The dependency of physiological processes on day- or nighttime has been obvious to humans since we are able to think. Because of this self-evidence the underlying mechanisms remained unexplored for a long time. It seemed clear that these changes are passive reactions to the sunlight. The first scientist who took a closer look into it was the French astronomer Jean Jacques Ortous de Mairan. He noticed that when a mimosa plant was placed in constant darkness in a cupboard, the leaves would still be rigid during daytime and droop at nighttime. This clearly meant that

sunlight is not needed as a signal for the leaves to get rigid, thus mimosa have an endogenous clock (1). Henri Louis Duhamel du Monceau even went further and placed the mimosa in a dark mine where also temperature would stay constant. He could still observe the regular movement of the leaves meaning that the rhythm is temperature compensated. This was very hard to belief at this time because it was known that a rise in temperature would also accelerate biological processes. In fact it took many more years to accept that circadian clocks are really temperature compensated (2). The first hint for circadian rhythms in mammals came from temperature rhythms analysis in monkeys (3). A behavioral endogenous circadian rhythm in mammals was first reported by Curt Richter who monitored the locomotor activity of rats in constant darkness (4). Although the field of circadian rhythms was developing quickly by then, the first clock gene mutant was only discovered in 1971 in *Drosophila melanogaster* (5). Since then, one highlight chased the other and even today great discoveries in the field are made at good pace. As mentioned by Gad Asher and Ueli Schibler "there is little risk that chronobiologists get bored within the next few years." (6)

3.1.3. The mammalian timekeeping system

The overall circadian timing in mammals is organized in a hierarchical manner. As a central clock the suprachiasmatic nucleus (SCN) in the hypothalamic region serves as a pacemaker, it is self-sustained, meaning that it can keep circadian oscillation without the aid of external cues. This master clock is adjusted to geophysical time by light inputs received by the retina in the eye and transmitted to the SCN via the retino-hypothalamic tract. The SCN, in turn, can synchronize clocks in other brain regions and in the periphery via neuronal and humoral signals. The SCN is therefore a so called zeitgeber (German word for "time giver"). Synchronized slave oscillators then regulate local rhythms in physiology and behavior. The SCN can be seen as the time distributor (Figure 1).

The SCN are paired nuclei located in the hypothalamic region right on top of the optic chiasm. Based on morphological differences the SCN can be subdivided in different parts, the two main ones being the "core" (ventrolateral) and the "shell"

(dorsomedial). The different regions of the SCN also have different pacemaking abilities, neuropeptide expression, response to environmental cues and rhythms to control. This is astonishing because the SCN is a very small structure consisting of not more than 20'000 cells in the mouse (7).

Friedrich K. Stephan and Irving Zucker in 1972 were the first scientist who showed that the specific deletion of only the SCN leads to behavioral arrhythmicity in rats (8) and Robert Y. Moore and Victor B. Eichler showed in the same year that SCN deletion disrupts the circadian rhythm of adrenal corticosterone. At this time it was assumed that the rhythm translation to the periphery is achieved by neuronal signals and this was confirmed experimentally by Shin-Ichi Inouye seven years later (9). That not only neuronal signals but also humoral signals can confer rhythmicity to the periphery was shown in another very elegant experiment. Rea Silver and co-workers implanted encapsulated SCN into SCN-lesioned hamsters thereby preventing neuronal outgrowth but permitting humoral diffusion. The rhythmicity of these animals was restored showing that diffusible signals are also involved in circadian entrainment of the periphery (10). Moreover, they implanted the SCN of a hamster containing a mutation leading to a shorter period length. Now, the restored hamsters adopted the period length of the donor hamsters. This phenomena of period length transplantation was already observed earlier by Martin Ralph delivering the final proof that the SCN is indeed the central timekeeper (11). The question remained, though, whether the SCN only controls behavioral and physiological rhythms or also clock gene expression oscillations. This was answered by analyzing the expression of a core clock gene in peripheral organs of SCN lesioned rats. The circadian rhythm of this gene was disrupted in the periphery (12). Moreover the expression was also abolished in leukocytes, which are not innervated, showing that also the control of circadian gene expression in the periphery is in part accomplished by humoral signals (13). It is, though, unlikely that these factors released by the SCN exert their function directly in the periphery. SCN cells are too low in numbers for for the ability to produce enough proteins to reach a concentration high enough in body fluid. It is more probable that they act locally in larger adjacent brain regions which then in turn produce high amounts of signaling molecules.

Several SCN derived diffusible molecules have been discovered since the year 2001: transforming growth factor (TGF)-α (14), prokineticin 2 (PK2) (15) and cardiotrophin

like cytokine (CLC) (16). All three molecules act as inhibitors of locomotion at different time windows leading to a robust and precise locomotor activity rhythm. Interestingly, no activators of locomotion have been found so far (Figure 1).

Slave oscillators, either cells in peripheral organs or cells in a culture dish, can keep the 24h rhythm in gene expression for only a few days without external input (17, 18). For a long time it was not clear whether this is due to a general dampening of the expression of core clock genes or if it is only a deception and in fact cells lose the synchrony and as a result are dephased. Two independent groups could show that in fact also peripheral oscillators are self-sustained (19, 20). The difference to the master clock is that there is no intercellular communication between individual cells to synchronize them. Therefore, and due to different period lengths of individual cells, a cell culture or peripheral organs dephase after a few days resulting in an apparent dampening of gene oscillation.

The SCN, however, is not the only pacemaker for the periphery. This was first suspected when Dorothy Krieger reported that SCN lesioned rats that are given one meal per day still display circadian rhythms in body temperature and adrenal hormone secretion (21). Moreover it is possible to completely uncouple the peripheral clocks (e.g. the liver) from the SCN by restricted daytime feeding. Normally, laboratory rodents have the food available at any time. When animals are only given food during the day when they normally sleep, the circadian rhythm of peripheral organs is inverted by 180° whereas the phase of the SCN remains the same (22). Meaning that for peripheral oscillators feeding is the more dominant zeitgeber than the SCN. It is therefore possible that the SCN entrains the periphery by controlling rest-activity cycles and thereby also feeding.

Body temperature, like scheduled feeding, has also been shown to serve as an indirect way of the SCN to synchronize the periphery as it is also controlled by sleep-wake cycles. However this zeitgeber is not very strong and dominated by the restricted feeding zeitgeber (23) (Figure 1).

As mentioned above, cells in an *in vitro* culture also possess a self-sustained clock, however this is only visible when the single cells are synchronized and oscillate in the same phase. High serum concentrations and multiple signals have been identified capable of resetting *in vitro* cultures. Sometimes the same signals are also used *in vivo*. One common feature in the synchronization of the cells by these signals is an

early induction of the central clock gene *Period 1*. The compounds comprise phorbol-12-myristate-13-acetate (PMA), Calcimycin, forskolin, dexamethasone, 12-O-Tetradecanoylphorbol-13-Acetat (TPA) and activate several different intracellular pathways such as Protein kinase (PK) A, PKC, cyclic adenosine monophosphate (cAMP), Ca^{2+}, mitogen activated protein kinase (MAPK), glucocorticoid receptor, G-protein-coupled receptors, glucose and retinoic acid receptor (18, 24-29). Some of these pathways, after a few steps, converge to a common one. The release of intracellular Ca^{2+} stores, the activation of PKC and the activation of G-protein-coupled receptors result in the activation of MAPKs and probably in the phosphorylation of cAMP response element binding protein (CREB). CREB in turn binds to CAMP response elements (CRE) in the *Per1* promoter resulting in induction of expression. It seems however that none of these pathways can fully account for the *in vivo* synchronization by the SCN or the *in vitro* synchronization by high serum concentration as blocking single signaling pathways doesn't influence the resetting properties (18, 25).

3.1.4. The intracellular clockwork in mammals

The molecular clockwork mechanisms in the SCN, the peripheral tissues and cell lines in culture are known to be very similar. They not only consist of transcriptional-translational feedback loops but are also very tightly regulated by post-translational modifications, such as phosphorylation, sumoylation and acetylation, chromatin remodeling and are also dependent on the metabolic state of the cell.

Transcriptional-translational feedback loops

Most of the circadian genes were first discovered in *Drosophila melanogaster* by mutagenesis studies. While searching for the mammalian homologues it became clear that the mammalian clock is similarly organized. However, as expected the clockwork is also more complex, for one gene in *D. melanogaster* even two or three were found in mammals. It became also clear that there is a high probability of finding circadian genes that are exclusive for the mammalian system, meaning that it is important searching for these directly in mammals.

Every mammalian cell has an autonomous molecular oscillator consisting of several core clock proteins acting in interlocked positive and negative feedback loops.

The two central elements of the positive loop are the basic helix-loop-helix (bHLH)-Period-Arnt-Single-minded (PAS) proteins BMAL1 (brain and muscle Arnt-like protein 1) and CLOCK (circadian locomotor output cycles kaput). These proteins heterodimerize and bind to *cis*-acting enhancer elements called E-boxes in the promoters of several genes including members of the negative feedback loop, the *Period* (*Per1* and *Per2*) and the *Cryptochrome* (*Cry1* and *Cry2*) family genes. A third *Period* gene (*Per3*) exists, however, it is more considered as a clock output gene than a core clock protein. There are differential reports about whether CLOCK/BMAL1 complexes remain bound to the DNA throughout the day or whether their E-box binding is circadian (30, 31). This might depend on the type of cells and the gene promoter. CRY and PER proteins in complex with casein kinase (CK) Iε and δ translocate back to the nucleus and inhibit the CLOCK/BMAL1 heterodimer and thereby also their own transcription. It appears that PER proteins are only modest inhibitors of CLOCK/BMAL1 activity, whereas CRY proteins are very efficient in inhibiting. This inhibition is most probably accomplished by stabilizing CLOCK and BMAL1 in an unphosphorylated and therefore inactive state (32). PER is important for recruiting CKI as CRY itself does not interact with the kinase. Moreover, CRY proteins are thought to mediate the nuclear import of the complex, although it has been reported that PER1 still reaches the nucleus in *Cry* deficient mice (27, 30). This may as well be cell type specific. In another regulatory loop, the orphan nuclear receptor *Rev-Erbα* is also activated via E-boxes by CLOCK/BMAL1. REV-ERBα, in turn, binds to the retinoic acid-related orphan receptor response (ROR) element in the *Bmal1* promoter to inhibit its transcription. While *Bmal1* mRNA falls, PER and CRY proteins rise leading to *Rev-erbα* repression via CLOCK/BMAL1 and therefore to de-repression of *Bmal1* (for review see (33)). Interestingly, simultaneously another nuclear orphan receptor, RORα, which contrarily activates *Bmal1* transcription, is activated. RORα binds to the same ROR element and therefore competes with Rev-Erbα (34). Additionally PER2 serves as positive drive for *Bmal1* as well. The mechanism is unclear but *Bmal1* mRNA levels are strongly reduced and arrhythmic in *Per2* mutant mice (35) (Figure 1).

Two other circadian proteins, differentiated embryo chondrocytes (DEC) 1 and DEC2, have been identified and analyzed in the last years. They can either directly bind to E-boxes and thereby act as inhibitors or in the presence of functional CLOCK/BMAL1 act as co-activators depending on the context of the E-box binding complexes (36, 37). They are thought to fine-tune the circadian cycles as the knock-down of both proteins only shows a mild circadian phenotype (38).

Even more recently, in 2007, another core protein, clock interacting protein circadian (CIPC), meeting all criteria for being a negative regulator was discovered. Its expression is also driven by E-boxes and by forming a ternary complex with CLOCK/BMAL1, CIPC inhibits their transcriptional activity (39).

A paralog of CLOCK, NPAS2, can replace CLOCK under some circumstances. Due to their overlapping function in the SCN, mice deficient in *Clock* or *Npas2* are still rhythmic although with a shorter period length. Therefore, deleting both genes leads to complete arrhythmia in constant darkness (40). Because peripheral clocks, in contrast to the central clock, do no longer oscillate in the absence of the *Clock* gene, it is clear that NPAS2 can only maintain rhythmicity in the SCN (41).

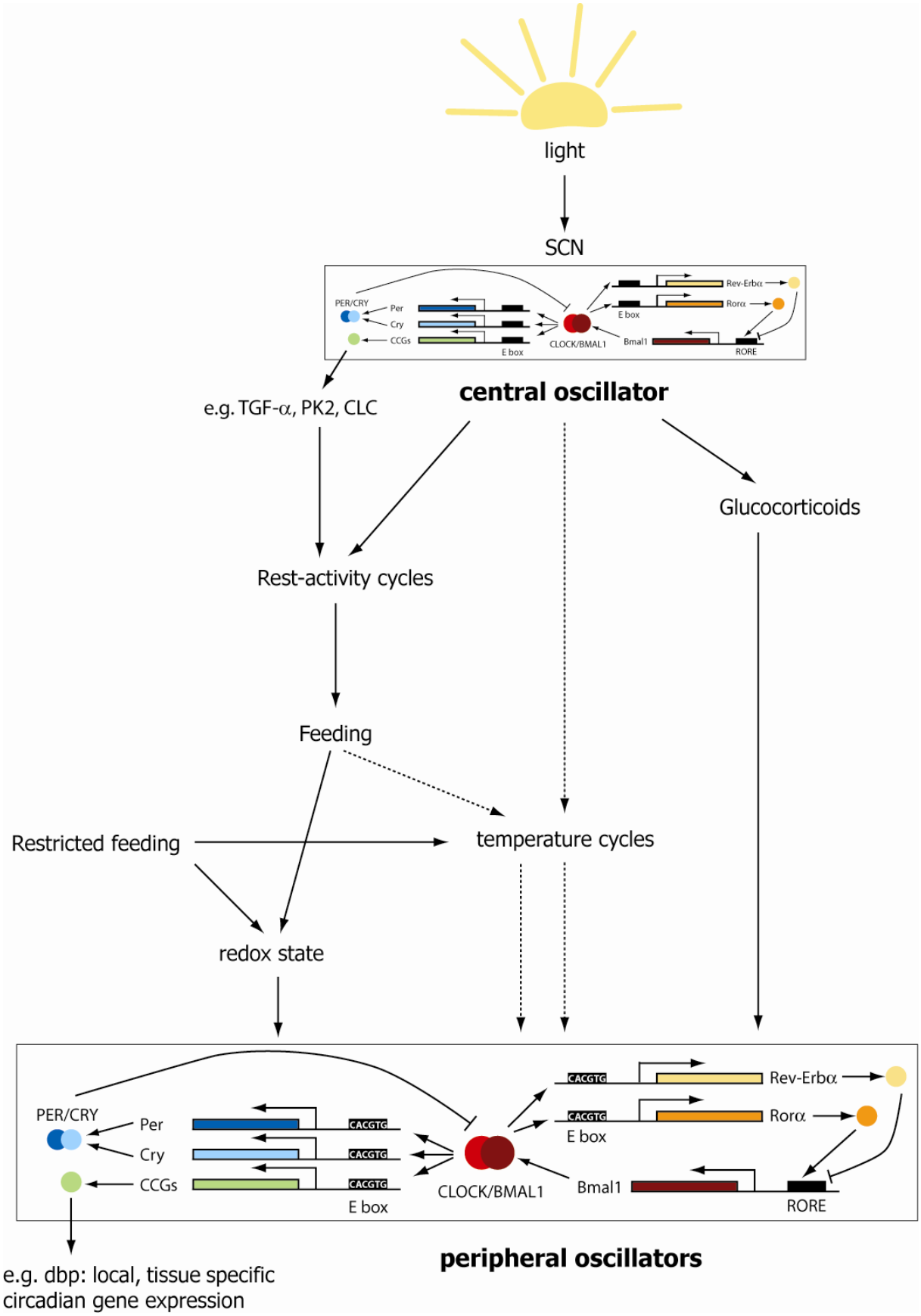

Fig.1: The mammalian timekeeping system (inspired by (42) and (43))

Chromatin remodeling

Chromatin remodeling is a well known and very important mechanism of transcriptional control. Histones can be modified with acetyl, methyl, phosphor, sumo or ubiquitin groups. It is not surprising that modifying histones also plays a dominant role in circadian transcriptional control.

Acetylation

The first hint of a rhythmical acetylation of histones was delivered by Jean-Pierre Etchegaray in 2003. He demonstrated that histones H3, but not H4, in the *Per1* and *Per2* promoters are acetylated with a circadian pattern resulting in transcriptional activation. The histone acetyl transferase (HAT) that accounts for this acetylation is p300, which he found to directly bind to CLOCK/BMAL1 heterodimers (44).

A few years later Jürgen Ripperger and Ueli Schibler very nicely showed that at the promoter of the clock output gene *D-element binding protein* (*Dbp*) the chromatin structure changes from a condensed to an uncondensed state in a circadian manner. This is achieved by a rhythmic change in histone modification. In that sense they showed that histone H3 acetylation at Lys 9 and the tri-methylation at Lys4 is circadian, and as expected the di-methylation of histone H3 at Lys 9 and HP1α binding to the DNA are exactly antiphasic (31).

Only two months past before Masao Doi made the great discovery that the central clock component CLOCK is not only a "boring" transcription factor but actually also an enzyme, namely a HAT. CLOCK is therefore capable of acetylating histones and this function is enhanced by its partner BMAL1 (45).

For Deacetylation it seems that the NAD+ dependent histone deacetylase (HDAC) sirtuin (SIRT) 1 is responsible. SIRT1 can form a complex with CLOCK/BMAL1 and deacetylate histone H3 (46). An involvement of SIRT1 mediated inhibition was already assumed earlier when it was found that the clock protein DEC2 associates with SIRT1 via the bHLH domain (37). DEC2, as described above, is a negative regulator that binds directly to E-boxes. It is therefore plausible that this inhibition is mediated via histone deacetylation by SIRT1.

Phosphorylation

Of all histone modifications, phosphorylation has the privilege to be directly linked to intracellular signaling pathways. Meaning that a kinase activated by an extracellular signal can directly contribute to chromatin remodeling. Additionally, histone phosphorylation can lead to an enhanced HAT-dependent histone acetylation (47).
In relation with circadian rhythms there is one interesting report revealing that a light pulse induces histone phosphorylation in the SCN in parallel with CREB phosphorylation and therewith *Per1* induction. The light induced kinases responsible for histone phosphorylation remain to be identified, however kinases of the AGC family are suspected (48).

Redox

By chance the group of Steven McKnight recognized that the ratio of reduced to oxidized nicotinamid adenine dinucleotide or the phosphorylated cofactor (NAD(P)H and NAD(P) respectively) has a great influence on the DNA binding of CLOCK and NPAS2. They showed that the heterodimeric interaction with BMAL1 is greatly stimulated by NAD(P)H. In contrast, the oxidized form even inhibited the interaction, leading to BMAL1 homodimers binding to Ebox sequences. These homodimers are incapable of activating transcription. Furthermore they report lactate dehydrogenase (LDH), an enzyme that catalyzes the reduction of pyruvate to lactate using the NADH cofactor, to be a direct target of CLOCK/BMAL1. Because LDH itself can also influence cellular redox potential and with this the DNA-binding abilities of its activator it can also be regarded as a clock component contributing to an additional negative loop (49).

Post-translational modifications

Transcriptional and translational feedback loops solely would not result in a 24 hours but rather in a much shorter rhythm of only a few hours. Although translational control may also contribute to a time delay, post-translational modifications have been the most well studied mechanisms for post-transcriptional control.

Many of the core clock genes are expressed circadian with the exception of *Clock*, which in many tissues does not cycle. However, when the cycling is destroyed by over-expression of these proteins, it does not eliminate the overt rhythm (50). This is due to the post-translational control leading to regulated destruction or activity of clock proteins (51).

Phosphorylation

Phosphorylation of clock proteins can have differential effects. It can alter subcellular location, activity, DNA-binding affinity and last but not least it can lead to protein ubiquitination and therefore degradation. It is therefore not surprising that most of the core clock proteins are phosphorylated with a circadian pattern (30).There is often no general statement possible that phosphorylation of a certain protein leads to a specific change because it depends strongly on which residues are phosphorylated by which kinase in what cell type. There is a set of kinases that have been shown to be involved in circadian rhythm generation.

CKI: The most important kinases involved in circadian rhythms are CKIε and CKIδ. A mutation of the CKIε gene in hamsters leads to period shortening of 4h. With the exception of *Clock* and *Bmal1* deletion which lead to arrhythmia the mutation or deletion of other core clock genes leads at maximum to a shortening or lengthening of 1.5h (52).

One important role of CKI is the control of protein degradation. The two negative regulators PER1 and PER2 are both phosphorylated by CKIε. Thereby a SKP1-Cullin1-F-box protein (SCF) E3 ubiquitin ligase containing β-transducin repeat containing protein (βTrCP) as an F-box protein is recruited. This subsequently leads to ubiquitinylation and degradation of these proteins by the proteasome pathway (53, 54). The CRY proteins are degraded by another SCF E3 ubiquitin ligase that contains FBXL3 as an F-box protein (55-57). Although it has not yet been shown that post-translational modification of CRY proteins are necessary for FBXL3 binding, it is very probable. CKI and also glycogen synthase kinase (GSK) 3β can phosphorylate CRY, and the function of phosphorylated CRY is unknown so far (58, 59).

The data about the influence of CKI on subcellular localization of PER is confusing. Depending on the cell type, phosphorylation of PER1 can lead to translocation into

the nucleus or into the cytoplasm (60, 61). CKI mediated phosphorylation of PER2, though, does not influence its subcellular localization.
CKI also positively regulates the activity of BMAL1 but not of CLOCK (58).
Intriguingly, CKI is constitutively expressed meaning that some other protein serve as regulators of the kinase. So far only one protein phosphatase (PP) could be identified to regulate CKI, namely PP5 (62). Although PP1, PP2A and PP2B are able to activate CKI *in vitro*, evidences that they are also physiological activators are missing. The role of phosphatases in the clock is therefore likely to be complex, because they may be involved in stimulating CKI activity and/or act in direct opposition to clock kinases by dephosphorylating PER proteins (see next chapter)

GSK-3β: As mentioned above GSK-3β also plays a major role in the circadian rhythm. The activity of this kinase is circadian *in vivo* in the SCN and *in vitro* in synchronized NIH 3T3. Inhibiting GSK-3β leads to a phase delay. It was shown to interact with and phosphorylate PER2 leading to nuclear translocation (63). Additionally it can also phosphorylate and thereby stabilize REV-ERBα (64) and finally, as already mentioned above, GSK-3β phosphorylates CRY2, targeting it to degradation when exiting the nucleus (59). GSK-3β itself is regulated by an inhibitory phosphorylation at a Ser residue, resulting in circadian activity cycles of this kinase (59)
MAPK and other kinases: The MAPK ERK and MEK have been shown to be activated in a circadian pattern in the chick pineal gland and the bullfrog retina, and that the inhibition of these kinases leads to a phase shift (65, 66). The same group describing the phase shift showed two years later that ERK phosphorylates BMAL1 and leads to inhibition of its activity. Three residues are phosphorylated (Ser-527/Thr-534/Ser-599) by the MAPK. Interestingly these residues are highly conserved in BMAL1 across vertebrates, raising the possibility that the same mechanism may take place in mammals (67). This is in contrast to phosphorylation of BMAL1 by CKI leading to an induction of activity, but this may be due to different phospho-acceptors of the two kinases. Together they may tightly regulate the activity status of BMAL1.
In mammals the MAPK p38 and ERK and the calcium/calmodulin dependent kinase II have been implicated in clock protein phosphorylation. However strong evidences for having a dominant role in the regulation of mammalian clocks are missing. Recently, PKC was described to phosphorylate CLOCK proteins, targeting it to the nucleus (68).

Obviously when kinases are active in some processes, phosphatases cannot be far away as well. Nevertheless, only little investigations about their role in circadian clocks have been conducted in mammals so far.

There is one study showing that PP1 binds to and dephosphorylates PER2, thereby opposing the effect of CKI. Hence, the protein is stabilized and prevented from degradation by the proteasome pathway (69).

Another group could show that PP5 indirectly regulate PER phosphorylation. They demonstrate the interaction of PP5 with CKI on one hand and with CRY proteins on the other hand. By binding to CKI it activates the kinase and therefore indirectly regulates the phosphorylation of PER. When CRY2 is added to this complex the PP5 mediated activation of CKI is inhibited. Thus CRY2 indirectly controls PER and maybe also its own phosphorylation (70).

Despite many reports about a role of PP2A in *Neurospora crassa* and *D. melanogaster* circadian control, until today no implications in the mammalian system have been found.

Sumoylation

Sumoylation is a small protein modification added by Small ubiquitin related modifier (SUMO) proteins. Like ubiquitin ligases, SUMO proteins can form polysumoylation chains. This modification, similar to phosphorylation, can then influence protein stability, localization and protein-protein interactions (For review see (71)). The first report about a role for sumoylation was provided by the group of Paolo Sassone-Corsi. They found that the core clock protein BMAL1 is sumoylated by SUMO1 with a circadian pattern and in a CLOCK dependent manner. BMAL1 protein mutated at the sumoylation site no longer displays circadian rhythmicity and was more abundant indicating that the modification destabilizes BMAL1 (72). A few weeks ago another group confirmed the sumoylation and hence the stabilization of BMAL1, however they claim that this is more likely to be achieved by SUMO 2/3 than SUMO1. Additionally they deliver evidences that after sumoylation BMAL1 is targeted to the nuclear body of the nucleus. Ultimately, BMAL1 is ubiquitinated, transactivated and transmitted to proteasomal degradation (73). This modification adds another degree to BMAL1 regulation. Probably it is a matter of time before sumoylation modifications are found on other clock proteins.

Acetylation

Very recently it was shown that CLOCK with its HAT activity is not only implicated in chromatin remodeling but also in acetylation core clock proteins. Indeed it was shown that BMAL1 is acetylated rhythmically with a peak in acetylation paralleling the downregulation of CLOCK/BMAL1 controlled genes. The acetylation is achieved by CLOCK and the repression is promoted by a facilitated recruitment of CRY1 (74). On the opposite SIRT1 is responsible for rhythmic deacetylation of BMAL1 (46).
That PER2 is also rhythmically acetylated was evidenced this year. The responsible acetyl transferase has not been discovered yet, but CLOCK and/or p300 are good candidates for it. What is clear so far is that SIRT1 is responsible for PER2 deacetylation and that this deacetylation leads to destabilization and degradation of the protein. It is hypothesized that PER2 is acetylated and ubiqutinated at the same residue so that SIRT1 would free the Lys to be ubiqutinated by removing the acetyl residue (75)

In my opinion in the next years research will come up with many other clock proteins discovered to be modified in many ways. Probably also other kind of modifications will be found, such as methylation, hydroxylation or glycosylation which have not been looked at so far.

3.1.5. The clock output

Once the slave oscillators are synchronized by the SCN, the local rhythms are entrained as well. It is thought that this is attained through first order clock controlled genes (CCGs). There is only a few rhythmic genes that are directly controlled by CLOCK/BMAL1 and regarded as clock output even though, all in all, microarray analysis of gene expression in different cell lines and mouse tissues have revealed that about 2-10% of the genes are in fact expressed with a circadian pattern, indicating that many of them are controlled indirectly. Moreover the expression is very tissue specific as only about 5% of the genes overlap between tissues. The first order CCGs are the direct way by which the core oscillation can be transduced to regulate downstream events in peripheral tissues, they are the

transcription factors controlling other cell specific transcription factors or enzymes (76-79).

The SCN output factors TGF-α, PK2 and CLC are considered to be CCGs. The proline and acidic amino acid-rich basic leucine zipper (PAR-bZIP) family member D-element binding protein is a prototype of a CCG. This PAR-bZip transcription factor has been involved in many aspects of metabolism of glucose (80), bile acid (81), or toxic compounds contained in food (82, 83). Its closely related genes thyrotroph embryonic factor (*Tef*) and hepatic leukemia factor (*Hlf*), although their direct regulation through CLOCK/BMAL1 has not been investigated so far, also play an important role in circadian physiological function. As mice lacking one or two of these genes only show mild phenotypes such as sleep disturbances and slight deviations in period length, it is thought that their functions are partly redundant (84, 85). When all three genes are knocked out, animals die of lethal audiogenic and spontaneous epileptic seizures (86). It is hypothesized that this is due to disequilibrium in neurotransmitter homeostasis. It was observed that these mice have a deficiency in pyridoxal kinase (*Pdxk*) expression. PDXK is involved in the vitamin B6 metabolism, turning it into its active form pyridoxal phosphate (PLP). PLP is a co-factor for many enzymes, among which many are involved in neurotransmitter metabolism (86). If the mice survive until the third month of age they no longer succumb to epileptic seizures but still exhibit high morbidity, rapid aging and die within one year. It is speculated that early aging and premature mortality arises from metabolic disorders caused by the absence of the PAR-bZIP proteins. In line with this, it has recently been shown that the triple knockout mice show a high deficiency in xenobiotic detoxification. Microarray data revealed that particularly the expression of genes involved in the detoxifying system is disrupted. It is nevertheless not clear whether these genes are direct targets of the PAR-bZIP transcription factors; anyway many of the affected genes harbor promoter sequences with strong homologies to PAR-bZIP-responsive elements (87).

3.1.6. What happens when the clock is out of time?

While in mice the different effects of single clock gene deletions or mutations are well known and have been analyzed in details, only a few consequences of such alterations have been identified in the human clock.

One of the most common is the familial advanced sleep phase syndrome (FASPS). People affected by FASPS go to bed and wake up about four hours earlier than the average. The disease is associated with a hypophosphorylated PER2 protein. Two mutations that lead to hypophosphoralytion have been found in FASPS pedigrees. The first one is a miss-sense mutation in the *Per2* gene within the binding site of CKIε and the second one is a mutation in the kinase itself leading to reduced enzymatic activity (88, 89). In the mirror case, known as delayed sleep phase syndrome people go to bed and wake up later than normal. A mutation in an auto-phosphorylation site of CKIε has been found to generate a kinase that is approximately 1.8 fold more active (90).

We all feel less active and motivated in wintertime. For some people it can even end up in depressions. These persons have a seasonal affective disorder (SAD). In recent years SAD could be associated with single nucleotide polymorphism (SNP) variants in *Per2*, *Bmal1* and *Npas2* with additive effects from combined risk genotypes (91). Other SNP could be associated with alcohol consumption (92).

The molecular clock is not only altered by clock gene mutations but can also be influenced by environmental changes. This is most apparently experienced when travelling around the world and suffering from jetlag or when working during night time and trying to sleep during day time. In both of these cases people are active and eat at a time that is not in concert with the internal clock. Indeed night-shift workers have poorer daytime sleep and reduced night time alertness and performance. Prominent health problems among shift workers include sleep disorders (which can become chronic), gastrointestinal disease, increased incidence of cardiovascular disease and possibly an increase in late-onset diabetes (93-95). At least for jetlagged persons this is only a temporary state, as the clock can shift approximately one hour per day and thereby synchronize to the new external environment. In contrast the shift-worker's clock is constantly in conflict with the environment.

Generally, a consequence of our today's lifestyle is already a disruption of the circadian rhythm, which not only results in sleep disturbances and depressions but has also been correlated to a higher susceptibility to cancer, especially breast cancer (96). Incidence of breast cancer in women increases proportionally with the amount spent working at night (97). Indeed, the circadian clock shares a number of conceptual and molecular similarities with the cell cycle (98). It has been shown that CLOCK/BMAL1 directly regulate a set of cell cycle genes such as *Wee1*, *c-myc* and *Cyclin D1* (99, 100). Moreover PER1 and PER2 are potent tumor suppressors in mice. Mice devoid of PER2 are more prone to malignant lymphomas (100). On the contrary, the ectopic expression of *Per2* results in growth inhibition and cell cycle arrest (101). These are all links between the clock and cancer, however the molecular mechanism is not understood so far.

3.2. INFLAMMATION

The response to a microbial pathogen can roughly be split into two phases. The first, the innate immune response is rapid and destructive and somewhat unspecific and self-limiting. The second, the adaptive response, is activated by the inflammation but takes a few days to be effective, it is very specific and includes memory function.
Cells involved in the innate immunity use invariant receptors called Toll-like receptors (TLRs) to detect and signal the occurrence of microbial infections (102, 103). By binding to TLRs, pathogens induce a highly coordinated process, which is commenced by the secretion of pro-inflammatory cytokines such as tumor-necrosis factor alpha (TNF-α), Interleukin (IL)-1beta, IL-6 and Interferons (IFNs). These cytokines alert and recruit other cells of the immune system such as neutrophils, macrophages and dendritic cells, which then help initiate the adaptive immune response. A hallmark of inflammation is the occurrence of fever, fatigue and weight loss (104).

3.2.1. Cytokine induced sickness behavior

Everyone who has experienced a bacterial or viral infection knows how it feels to be sick. We then feel tired, listless and irritable, loose appetite and interest in social

activities, have fever and nausea and our sleep is often fragmented. Collectively these symptoms are referred to as "sickness behavior". These changes make perfectly sense; they are a highly organized strategy of the organism to fight infections and contribute to the recovery. The organism, thereby, prevents the occurrence of those activities that are metabolically expensive and favoring those that decrease heat loss and increase heat production. However, these symptoms are also well known to have a negative impact on the quality of life of patients with chronic inflammatory disorders.

As soon as the sequences of the pro-inflammatory cytokines IL-1, IFN-α and TNF-α were identified and the proteins expressed in recombinant forms, they were tested in patients with a variety of cancers or, in the case of INF-α, with infectious diseases. The systemic administration of these cytokines was soon recognized to give the same side effects as after an infection; they lead to sickness behavior.

The cytokines produced upon an infection or injected in the periphery also act on the brain. There are several pathways of communication. The fastest one involves afferent nerves such as the vagal nerves that are activated by the locally produced cytokines. Animals injected with LPS, IL-1 or TNF-α intraperitoneally show the full spectrum of sickness behavior, however upon vagotomy these symptoms are reduced or disappear (105-107). It is thought that the stimulation of the vagal nerves leads to IL-1 and subsequently also TNF-α and prostaglandin of type E expression in the brain, which in turn act on specific neural substrates to induce sickness behavior (108). Vagotomy, however has no effects when cytokines are injected subcutaneous or intravenously (109, 110) indicating that other routes are employed to communicate with the brain. Indeed, a second slower pathway exists. Macrophage-like cells residing in the circumventricular organs and the choroid plexus, near the blood brain barrier, express TLRs and cytokine receptors and can thereby respond to circulating pathogens or cytokines (111, 112). The result of both pathways is the production of pro-inflammatory cytokines by microglial cells in the brain. Presumably, activation of the neural pathway sensitizes target brain structures for the production and action of cytokines diffusing from the circumvetricular organs and the choroid plexus. (113-115).

3.2.2. Tumor necrosis factor-α signaling

Already more than one century ago it was discovered that antitumoral agents are released after a pathogen infection (116). This knowledge led to the idea to cure tumors by the use of bacterial extracts (117). It needed another 70 years to realize that the effect of bacterial extracts is not direct but mediated by a factor that was designated as TNF-α (118). After another 20 years, TNF-α was cloned and analyzed. The structure and function revealed a close homology to lymphotoxin-α (119). Almost simultaneously and independently cachectin was discovered as a mediator of cachexia in patients infected with trypanosomes. Immediately, it became clear that TNF-α and cachectin are in fact one and the same molecule (120).

TNF-α forms homotrimers and is primarily produced as a transmembrane protein. This membrane-integrated form can be cleaved by the metalloprotease TNF-α converting enzyme (TACE) to release the likewise homotrimeric soluble TNF-α (121, 122).

Two receptors forward the signal into the cell: TNF receptor (TNFR) 1 and TNFR2. Whereas TNFR1 is constitutively expressed, TNFR2 is highly regulated and preferentially found on endothelial cells and cells of the immune system. Additionally TNFR2 can only be fully activated by membrane bound TNF-α (123). The extracellular domain of both receptors can be cleaved resulting in fragments with neutralizing potential (124).

TNF-α binds to the already pre-assembled receptor. Interestingly neither receptor contains any enzymatic activity. Instead, the intracellular parts harbor protein interacting domains.

By TNF-α binding to TNFR1 the inhibitory protein silencer of death domain (SODD) is released from the intracellular tail and replaced by the adaptor protein TNF receptor-associated death domain (TRADD). The three adaptor proteins receptor-interacting protein (RIP), TNFR-associated factor 2 (TRAF2) and Fas-associated death domain (FADD) are recruited as well and are responsible of initiating the intracellular signaling. The first event in the apoptosis cascade is recruitment of caspase 8 to FADD where it becomes activated. TRAF2 activates extracellular signal-regulated kinase kinase kinase 1 (MEKK1) or apoptosis stimulated kinase 1 (ASK1), activating a kinase cascade which will result in the activation of c-jun NH_2-terminal kinase (JNK).

The phosphorylation and heterodimerization of c-jun to form the transcription factor activator protein-1 (AP-1) leads to the transcription of proteins involved in proliferation, differentiation and induction, as well as prevention of apoptosis (125). Besides JNK, TRAF2, MEKK1 and ASK1 together with RIP have also been implicated in the activation of p38 MAPK (126). The activation of both kinases is only transient, but show prolonged action under apoptotic conditions. The p38 MAPK signaling pathway is a mediator of inflammatory processes by inducing the production of IL-1 and IL-6. The mechanism, however, is not known. The best studied arm of TNFR1 signaling is the nuclear factor kappa B (NF-κB) pathway, which involves the adaptor protein RIP. In quiescent cells NF-κB is retained in the cytoplasm by the inhibitor of κB (IκB). Upon stimulation, the multiprotein IκB kinase (IKK) complex is recruited to the receptor, activated in a RIP dependent manner and then phosphorylates IκB. IKK consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NF-κB essential modulator (NEMO). Upon phosphorylation IκB is ubiquitinated and degraded allowing NF-κB to enter the nucleus. There it binds to NF-κB site and activates the transcription of anti-apoptotic as well as pro-inflammatory proteins.

The signaling pathway of TNFR2 is less clear. In contrast to TNFR1, TNFR2 lacks an intracellular death domain (DD). It instead can directly recruit TRAF2 and activate the NF-κB pathway. The activation of TNFR2, unlike TNFR1, may promote cell activation, proliferation and migration. However, TNFR2, under some circumstances, may contribute to TNFR1 signaling. At low TNF-α concentrations TNFR2 passes its ligand to TNFR1 (127, 128).

TNF-α can have both harmful and beneficial activities. The transmembrane expression indicates that this molecule is meant to act locally. However, under non-physiological conditions, TNF-α is released and acts on various cell types to mediate its systemic effects. Hence, neutralizing TNF-α can be helpful to cure some diseases but serious harmful side effects have been observed as well. These comprise tuberculosis infection, demyelinating syndromes and systemic lupus erythaematosus-like reactions (129). (130-132).

3.3. AIM OF THE PROJECT

The symptoms of sickness behavior, such as fatigue, reduced appetite, sleep disturbances, provoked by cytokines have a negative impact on patients with chronic inflammatory disorders but not much can be done to alleviate these symptoms. The mechanisms by which the periphery talks to the brain to induce sickness behavior have only been explored in recent years and our understanding of communication pathways is not complete. Sickness behavior including less activity, fever, listless (kind of depression) and also less eating, has been described to be directly transduced to the brain from an infection in the periphery by the vagal nerves or humoral signals. We, however, think that cytokines not only talk to the brain by the two known pathways but also act on peripheral organs to influence the circadian clock and thereby alter metabolism and activity. These alterations may have an indirect impact on the brain resulting in alterations of rest-activity cycles, feeding behavior and temperature.

As mentioned above the central and peripheral clocks can be reset by a variety of different chemical compounds and hormones. Several studies report that also some cytokines can modulate the clock: IL-6 (133), IFN-α (134, 135), IFN-γ (136), as well as the hormone Prostaglandin E2 (137). It is important to know the impact of cytokines on the circadian system and to elucidate the pathways by which cytokines modulate the molecular clock. Only that way it will be possible in the future to intervene in the cascade to alleviate specific side symptoms without interfering with the entire immunological process.

So far, no reports can be found about the pro-inflammatory cytokine TNF-α, although it plays a pivotal role in the cytokine induced sickness behavior. Therefore, the aim of my thesis was to observe the effects of TNF-α on the molecular clock in an *in-vitro* system with a fibroblast cell line. Using several compounds interfering with different arms of the TNFR1 and TNFR2 signaling pathway, our ambition was to define the route leading to clock gene expression variations.

4. RESULTS

4.1. TNF-α SUPRESSES THE EXPRESSION OF CLOCK GENES BY INTERFERING WITH E-BOX MEDIATED TRANSCRIPTION

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My contributions to this work: I have contributed to Fig. 1 and 3.

TNF-α suppresses the expression of clock genes by interfering with E-box mediated transcription

Gionata Cavadini*, Saskia Petrzilka*, Philipp Kohler*, Corinne Jud[†], Irene Tobler[‡], Thomas Birchler*, and Adriano Fontana*

*Division of Clinical Immunology, University Hospital Zurich, Haeldeliweg 4, CH-8044 Zurich, Switzerland; [†]Institute of Biochemistry, University of Fribourg, Rue du Musée 5, CH-1700 Fribourg, Switzerland; [‡]Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstr. 190, CH-8057 Zurich, Switzerland
G.C. and S.P. contributed equally to this work; T.B. and A.F. contributed equally to this work

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Summary

Production of TNF-α and IL-1 in infectious and autoimmune diseases is associated with fever, fatigue and sleep disturbances which are collectively referred to as sickness behavior syndrome. In mice TNF-α and IL-1 increase non-rapid eye movement sleep. Because clock genes regulate the circadian rhythm and thereby locomotor activity and may alter sleep architecture we assessed the influence of TNF-α on the circadian timing system. TNF-α is shown here to suppress the expression of the PAR bZip clock-controlled genes *Dbp, Tef* and *Hlf* and of the period genes *Per1, Per2,* and *Per3* in fibroblasts *in vitro* and *in vivo* in the liver of mice infused with the cytokine. The effect of TNF-α on clock genes is shared by IL-1β, but not by IFN-α, and IL-6. Furthermore, TNF-α interferes with the expression of *Dbp* in the suprachiasmatic nucleus and causes prolonged rest periods in the dark when mice show spontaneous locomotor activity. Using clock reporter genes TNF-α is found here to inhibit CLOCK-BMAL1 induced activation of E-box regulatory elements-dependent clock gene promoters. We suggest that the increase of TNF-α and IL-1β, as seen in infectious and autoimmune diseases, impairs clock gene functions and causes fatigue.

Introduction

In microbial infections, host defense mechanisms activate the innate and adaptive arms of the immune response. Microbial recognition by Toll-like receptors (TLR) expressed by macrophages and dendritic cells leads to the activation of signal transduction pathways with induction of various genes including IL-1, TNF-α, IL-6, and IFN-α/β (1-3). These cytokines mediate the acute–phase response, which is a systemic generalized reaction characterized by fever, fatigue, and weight loss, an increase in the number of neutrophils, and the induction of synthesis of acute-phase proteins in the liver with increased haptoglobin, antiproteases, complement

components, fibrinogen, ceruloplasmin, and ferritin in the blood (4). An acute phase response with a dose-dependent state of lethargy and severe fatigue has been described in cancer patients treated with TNF-α (5). A link between production of TNF-α and daytime fatigue has also been suggested in rheumatoid arthritis (RA) and in the obstruction sleep apnea syndrome (OSAS). Inhibition of TNF-α by soluble TNF-receptor p75 improves disabling fatigue in patients with RA (6). A TNF-α-308 (A-G) single-nucleotide polymorphism and elevated TNF-α serum levels have been described in OSAS (7, 8). Moreover, neutralization of TNF-α reduces daytime sleepiness in sleep apneics (9). Direct effects of TNF-α on spontaneous sleep are also shown in animal studies. i.v., i.p., or intracerebroventricular injections of TNF-α or IL-1 enhance nonrapid-eye movement (NREM) sleep (for reviews see refs. 10 and 11). This increase in NREM sleep is independent of the fever-inducing capacity of these cytokines (12). Although there is good evidence for TNF-α and IL-1 as mediators of altered sleep-wake behavior, the underlying mechanisms remain elusive.

The regulation of sleep depends on a circadian control and a homeostatic drive (13, 14). The circadian influence is provided by the suprachiasmatic nuclei (SCN) of the hypothalamus being entrained by light stimuli to the environment. This self-sustaining circadian pacemaker uses a molecular mechanism similar to the one used in subsidiary oscillators present in any type of cell in the organism. The molecular clockwork involves the transcriptional repressor genes *Per1*, *Per2, Cry1*, and *Cry2*, as well as the transcriptional activators *Bmal1* and *Clock*. The heterodimerized transcription factor BMAL1:CLOCK activates *Per* and *Cry* gene transcription by binding to E-box motives in their promoters. PER and CRY proteins inhibit BMAL1:CLOCK complexes, thereby inhibiting their own gene expression. This feedback-loop mechanism generates circadian oscillations of *Per* and *Cry* expression. The same positive and negative regulatory components also govern the rhythmic expression of the nuclear orphan receptor *Rev-Erbα*, which in turn represses the transcription of *Bmal1* through direct binding to a REV-ERBα response element in the *Bmal1* promoter. Thereby, REV-ERBα interconnects the cyclic expression of positive- and negative-loop members (for reviews, see refs. 15 and 16). The targeted inactivation of *Bmal1* showed that this gene is indispensable for the maintenance of

circadian functions (17). *Clock*-deficient mice show robust circadian patterns of locomotor activity with period lengths shortened by only twenty minutes; *Dbp* mRNA rhythm, however, was severely blunted in both the SCN and the liver (18). The deletion of the clock-controlled genes (CCG) PAR bZip transcription factors *Dbp*, *Tef,* and *Hlf* only moderately affects the circadian clock but leads to pronounced disturbances of locomotor activity. In the present study, our objective was to examine (*i*) whether and how TNF-α influences the circadian timing system and modulates the expression of clock genes and CCGs, and (*ii*) whether TNF-α affects locomotor activity, rest time, and periodicity in mice.

Results

Suppressed expression of clock genes in TNF-α treated synchronized fibroblasts

To determine whether TNF-α interferes with circadian gene expression, we used NIH 3T3 fibroblast cultures synchronized by a 2-h serum shock; this system elicits a well described circadian expression of central clock genes and CCGs for at least three cycles (19). We analyzed serum shocked fibroblasts challenged over time with or without TNF-α (10 ng/ml). RNA was extracted every 4 h thereafter up to Zeitgeber time (ZT) 36 and analyzed by using quantitative real-time RT-PCR methods. Within the first 12 h, the course of the examined clock gene expression was not altered by TNF-α treatment. However, around the peak (ZT 20-28), TNF-α strikingly suppressed the expression of the central clock genes *Per1*, *Per2*, and *Per3* and of the CCGs *Dbp, Tef*, and *Hlf.* Peak expression of *Rev-erb*α mRNA at ZT 20 was slightly increased, whereas *Bmal1* expression was not affected by TNF-α. *Clock* expression was slightly increased. For all genes, TNF-α did not affect the phases but rather attenuated the amplitude of expression (Fig. 1A). Therefore, we assessed the extent of clock gene expression at their main peak at ZT 24. TNF-α induced a significant reduction of gene expression for *Per1, Per2, Per3, Dbp, Tef*, and *Hlf* genes*. Rev-Erb*α expression, as assessed at its peak of expression at ZT 20, remained unchanged by TNF-α, as

did *Clock* (Fig. 1B). We extended the time of observation over a second circadian cycle and analyzed rhythmic expression up to 60 h. In this, emphasis was placed on *Dbp* expression, because its amplitude was the most affected by TNF-α. Rhythmicity and period length of *Dbp* expression were not changed by TNF-α, but its amplitude was severely suppressed at both peaks (Fig. 1C). To determine whether the TNF-α-mediated suppression is reversible, we washed out TNF-α after the first cycle (at ZT 24) and analyzed the second peak at ZT 48. By washing out TNF-α, the second peak of expression of *Per1* and *Per2* was restored, and *Dbp* expression increased compared with the control where TNF-α still led to reduced peak expression. The expression of *Clock* was not affected when extending the time of TNF-α treatment to 48 h (Fig. 1D). Moreover, the suppression of *Dbp*, *Per1*, and *Per2* gene expression in synchronized NIH 3T3 fibroblasts is clearly dose-dependent, being most significantly affected by concentrations > 1 ng/ml TNF-α. The expression of *Clock* remained unchanged irrespective of the dose of TNF-α (Fig. 1E).

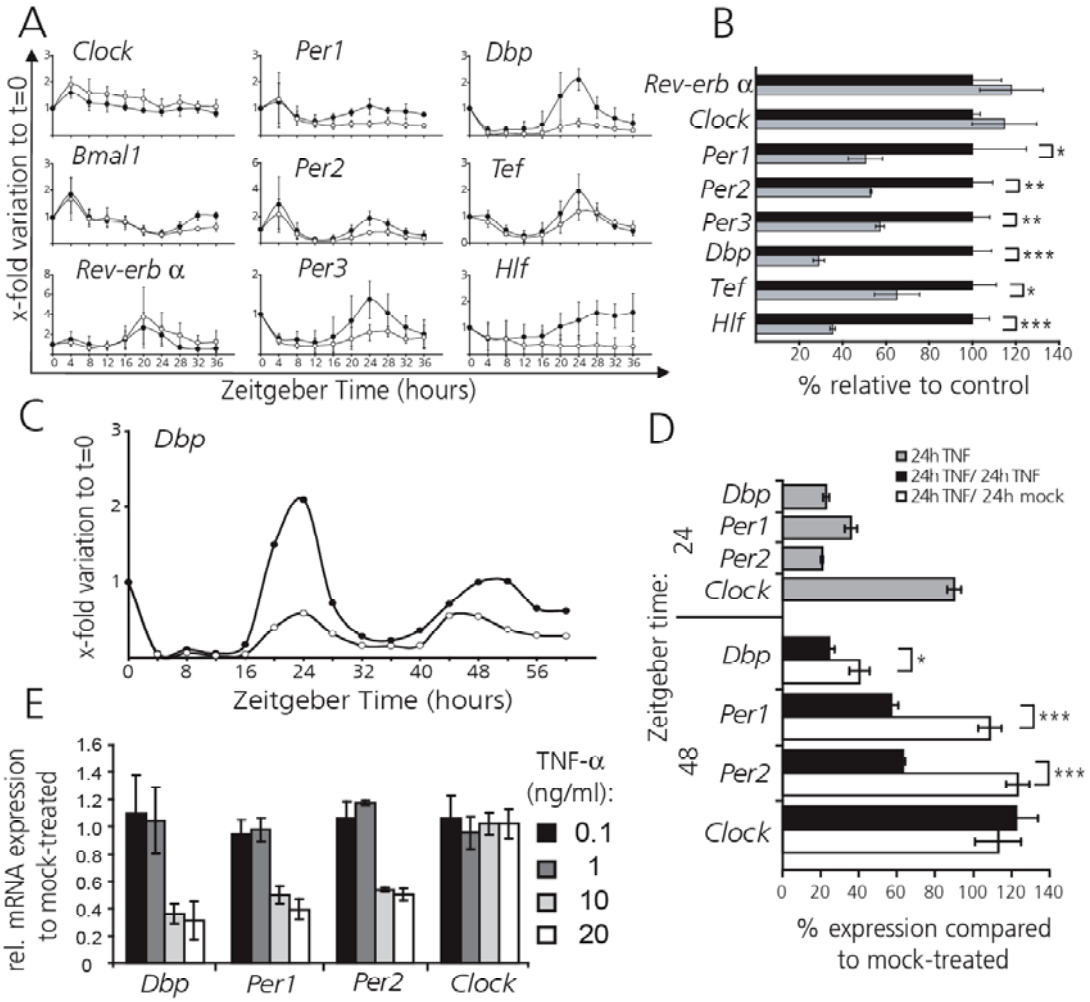

Fig. 1. TNF-α impairs expression of clock genes in synchronized NIH 3T3 fibroblasts. (A) TNF-α attenuates circadian *Per1/2/3* and PAR bZIP family (*Dbp*, *Tef*, and *Hlf*) gene expression. After serum shock (ZT 0-2), cells were kept in serum-free medium with TNF-α (10 ng/ml; open circles) or without the cytokine (filled circles) and analyzed every 4 h with quantitative real-time RT-PCR. Results are shown as x-fold variations to nonsynchronized fibroblast cultures at ZT 0; three independent experiments; mean values ± SD. (B) *Per* and PAR bZIP family genes are significantly down-regulated at the 24-h peak (TNF-α: gray bars; controls: black bars), whereas *Clock* and *Rev-Erbα* are not affected. Expression of *Rev-Erbα* was assessed at its peak at ZT 20. Data show one representative experiment done in triplicate (mean ± SD) of four experiments. (C) The amplitude of *Dbp* expression in serum-shocked NIH 3T3 fibroblasts was attenuated during 60 h in the presence of TNF-α (open circles) compared with controls (filled circles). (D) Withdrawal of TNF-α after the first peak at ZT 24 shows that the suppression of *Dbp, Per1, and Per2* at the second peak at ZT 48 is reversible. After serum shock, cells were treated with TNF-α for 24 h (gray bars), or for 48 h with or without a withdrawal of TNF-α after 24 h (white and black bars, respectively). Gene expression was compared to mock-treated cells at the respective ZT (100% expression). Data show one representative experiment done in triplicates (mean ± SD) of three experiments. (E) The suppression of the expression of *Dbp, Per1*, *and Per2* in synchronized fibroblasts at ZT 24 is dose-dependent being significant ($P < 0.005$) at doses higher than 1 ng/ml TNF-α. Data show the mean ± SD of three independent experiments performed in triplicates. For BDE, we used the independent-sample *t*-test ; *, $P \leq 0,05$; **, $P \leq 0,005$; ***, $P \leq 0,0005$.

IL-1β but neither IFN nor IL-6, shares with TNF-α the effect to down-regulate clock genes

Besides TNF-α other cytokines are produced by TLR-activated macrophages and dendritic cells that have been implicated in the sickness behavior syndrome, namely the proinflammatory cytokines IL-1, IL-6, and IFN-γ and type I IFN cytokines IFN-α and -β (20). Therefore, we examined whether the effect of TNF-α is clock genes is a unique property of TNF-α or is shared by other cytokines. Besides TNF-α also IL-1β suppressed the expression of both, *Dbp* and *Per3* (Fig. 2). The expression of *Dbp, Per1, Per2*, and *Per3* was not inhibited by treatment of synchronized fibroblasts with IL-6 and IFN-α. IFN-γ induced a significant, albeit minor, suppression of *Dbp* expression.

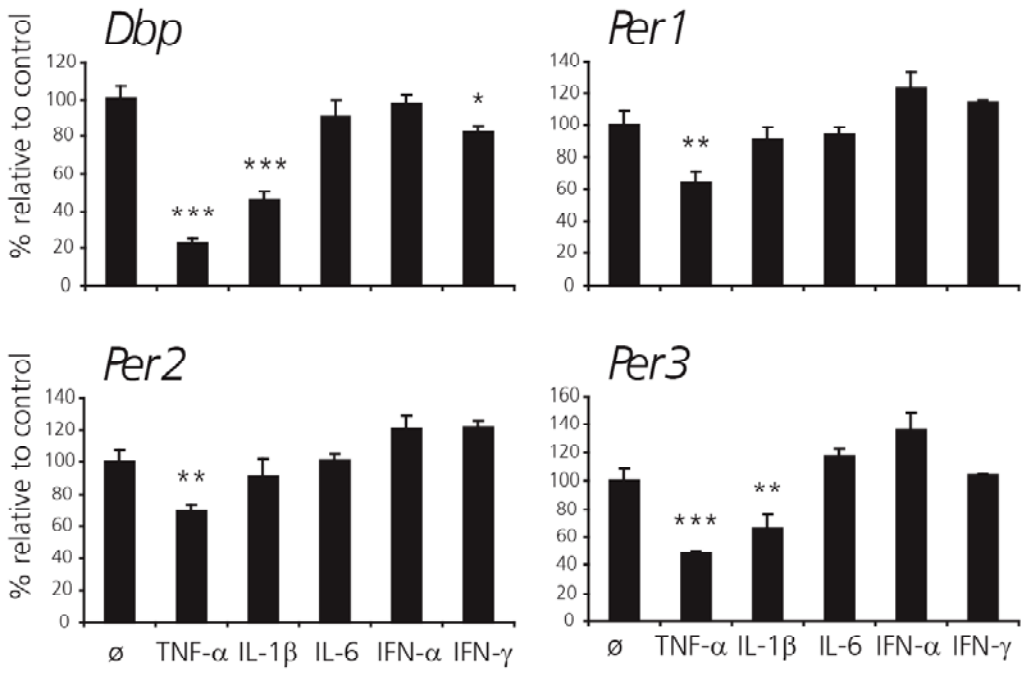

Fig. 2. Cytokine effects on *Dbp*, *Per1*, *Per2*, and *Per3* in N1H 3T3 fibroblasts. Confluent cells were synchronized with 50% horse serum for 2 h (ZT 0-2). After serum shock, cells were kept in serum-free medium with TNF-α (10 ng/ml), IL-1β (10 ng/ml), IL-6 (10 ng/ml), IFN-α (10 ng/ml), IFN-γ (20 ng/ml) or without cytokines and analyzed at ZT 24 with quantitative real-time RT-PCR. Results are shown as percent of expression to noncytokine-treated fibroblast cultures; one representative experiment of three; mean values of triplicates ± SD; independent sample *t*-test; *, $P \leq 0,05$; **, $P \leq 0,005$; ***, $P \leq 0,0005$.

TNF-α interferes with E-box-dependent transcription of clock genes

Circadian transcriptional activation of Per genes and of the PAR bZip transcription factor genes *Dbp*, *Tef*, and *Hlf* is thought to depend on the binding of the heterodimer BMAL1:CLOCK to the canonical or non-canonical E-box, a basic helix-loop-helix transcription factor-binding site ideally harboring the sequence CACGTG (21). In our study, TNF-α affected suppression only of the transcription of those clock genes harboring an E-box upstream of the transcription initiation site (*Per* genes and *Dbp, Tef*, and *Hlf*). No suppression was observed on *Bmal1* and *Clock* expression, their transcription not being E-box-dependent. Thus, TNF-α may interfere with E-box-mediated transcriptional activation. To test this hypothesis, we performed transient transfections of NIH 3T3 cells with luciferase reporter genes under the control of the native 3- or 1.7-kB promoter sequences of mouse *Per1* and *Per3,* respectively, and of the 1-kB promoter of *Bmal1* (Fig. 3A). Consistent with the gene expression studies, *Per1* and *Per3* promoter activity was suppressed after TNF-α administration but not that of the *Bmal1* promoter, which is devoid of E-box elements (Fig. 3B). To specify the possible effect on E-boxes, we performed assays using NIH 3T3 cells stably transfected with a luciferase reporter plasmid consisting of three E-boxes within 2.0 kb of the 5′ flanking region of the mouse *Per1* gene with their immediate flanking sequence linked together and joined to the thymidine kinase promoter (22) (Fig. 3A). Again, treatment with TNF-α reduced luciferase activity by 45%, corresponding to the basal activity of the basic pGL3-TK vector without E-boxes (Fig. 3C). An E-box reporter construct of the *Dbp* gene (Dbp-E-box857; Fig. 3A) that was cotransfected with plasmids expressing CLOCK and BMAL1 proteins showed expression reduced by 67%, indicating that overexpressed CLOCK and BMAL1 are still efficiently blocked. In contrast, with the mutated E-box (ACCAGT instead of CACATG) reporter construct (Dbp-857 mut), the activity was significantly derepressed (Fig. 3D). We suggest therefore that TNF-α suppresses E-box-mediated transcription of clock genes. Thus, selective down-regulation of clock genes or CCGs is likely to depend on the presence of E-box elements in the respective genes.

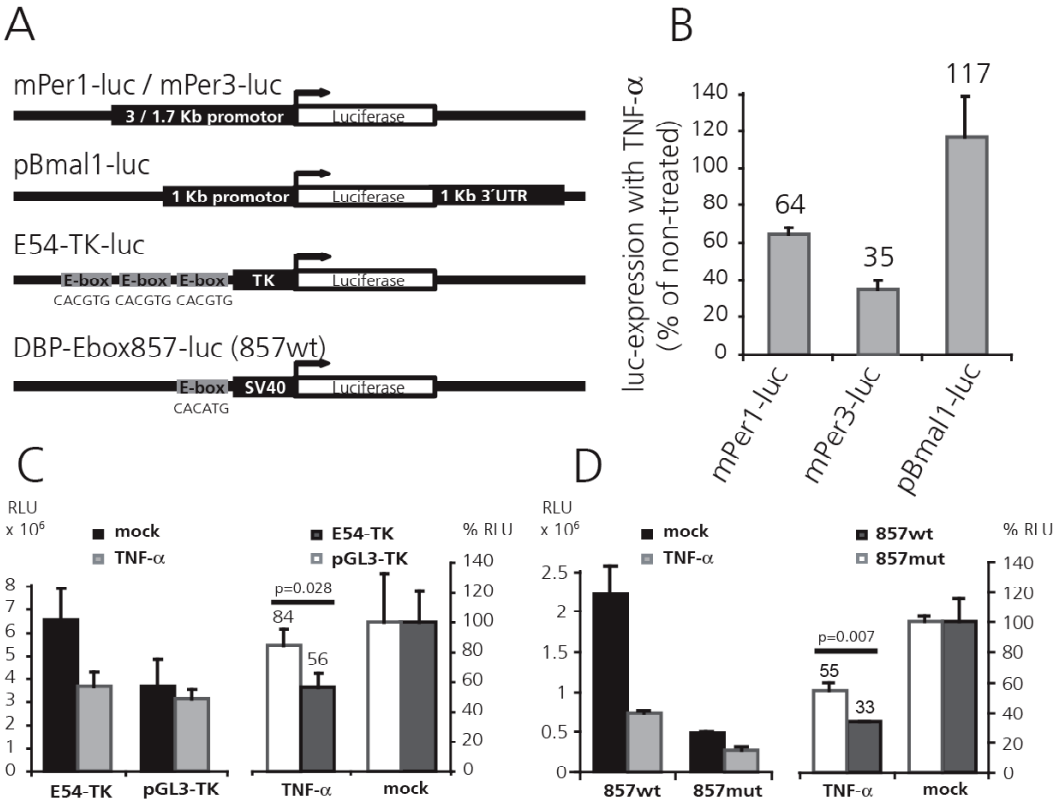

Fig. 3. TNF-α suppresses E-box-mediated transcription of clock genes. (A) Schematic representation of the luciferase reporter genes used. mPer1-luc (3-kb promoter fragment) and mPer3-luc (1.7 kb promoter fragment) contain promoter sequences upstream of their genes in the pGL3basic vector. pBmal1-luc is regulated by a 1-kb promoter fragment and a 1-kb sequence of the 3'UTR from *mBmal1* gene. E54-TK consists of the three E-boxes of the mouse *Per1* gene and their immediate flanking sequences in front of a TK promoter. DBP-Ebox857-luc contains one of four E-boxes of the *Dbp* promoter regulating the activity of a SV40 promoter. (B) Native clock gene promoters bearing an E-box are affected by TNF-α. Percent luciferase expression of mPer1-luc, mPer3-luc, pBmal1-luc transfected NIH 3T3 cells after treatment with 10 ng/ml TNF-α overnight compared to untreated controls (100% luciferase expression) is shown. (C) E-boxes of *mPer1* are affected by TNF-α treatment. TNF-α significantly reduces E54-TK-dependent luciferase expression but not pGL3-TK lacking E-boxes and flanking sequences as shown by raw data (RLU, Relative Light Units; Left) and percent inhibition (Right). (D) The E-box of *Dbp* gene at position +857 is repressed by TNF-α even when CLOCK and BMAL1 are overexpressed. Cotransfection with CLOCK and BMAL1 leads to four to five times higher relative luciferase activity of 857wt compared with the mutated E-box vector (857mut) indicating that the interaction of CLOCK:BMAL1 with the E-box is functional. Luciferase activity is efficiently suppressed by overnight treatment with TNF-α as shown by raw datas (Left) and percent inhibition (Right). TNF-α leads to a higher repression in 857wt than in 857mut. For all assays, mean ± SD of triplicates from one representative experiment of three; independent sample *t*-test.

Reduction of clock genes and CCGs in TNF-α-treated mice

To assess the effect of TNF-α on the expression of clock genes *in vivo*, C57Bl/6 mice were constantly infused with TNF-α (1,5 μg/day or 0,075 mg/kg per day) via an osmotic minipump inserted s.c. on the back of the mice over a period of 7 days (see Materials and Methods). The dose chosen is ≈10-fold lower than the dose used to induce a septic shock-like disease in rats (0,7 mg/kg, administered intravenously) (23). Histopathology revealed no evidence for TNF-α-induced vascular damage, hemorrhages, or inflammation in the liver, lung, and kidney based on morphological criteria and on the expression of heme oxygenase (HO-1), a marker for oxidative stress. Macrophages of mice infused with TNF-α showed signs of being activated in the liver and kidney (supporting information (SI) Fig. 6). We found TNF-α serum concentrations at 43 (±10.9) pg/ml at day three in TNF-α-treated mice compared with 10 (±0.8) pg/ml in controls (SI Fig. 7). In mice with experimental septic shock the respective value for TNF-α exceeds 10 ng/ml (24). In controls, osmotic pumps were filled with diluent (PBS, 0.1% BSA). Total RNA was extracted from the liver of mice treated with TNF-α or control; expression of clock genes was tested at day three at ZT 11.5 and ZT 15.5 (ZT 0 = 6 a.m. lights on, ZT 12 = 6 p.m. lights off), when we encountered maximal inhibition of locomotion (Fig. 5A). As shown for fibroblasts *in vitro*, TNF-α also suppressed the expression of *Dbp*, *Tef*, and *Hlf*, and *Per3*, but not of *Bmal1* and *Clock* (Fig. 4). Although *Per1* and *Per2* were down-regulated, this was not significant. However, at a later time point (ZT 15.5), when *Per2* normally reaches its peak of expression, significant suppression of *Per2* was observed (Fig. 4 Lower). In contrast to the fibroblast data, we found that TNF-α suppressed the expression of *Rev-Erbα.* TNF-α induced reduction of expression of the *Per* genes and of PAR bZip transcription factor genes is likely to influence the expression of clock output genes. Among the genes that are positively regulated by DBP is the liver-specific albumin gene (25). The capacity of TNF-α to interfere with *Dbp* mRNA expression is congruent with the observation of a decrease in albumin serum concentration by 41% in TNF-α-treated mice compared with controls (SI Fig. 8). TNF-α has already been described as inhibiting albumin synthesis in liver cells, but the mechanisms remain elusive (26). Taken collectively, these data provide

evidence that the suppressive role of TNF-α on *Dbp* is associated with impaired activation of its target gene. TNF-α-treated mice also show higher endogenous expressions of IL-1β and TNF-α in the liver (both genes are well known to be induced by TNF-α (28)); the level of expression of these cytokines correlates with each other (SI Fig. 9). Furthermore, the increased expression of the cytokines in the liver is associated with a decrease of the CCG *Dbp*, *Hlf*, and *Tef*; the extent of the decrease of these PAR bZip transcription factors fits to the TNF-α expression in individual mice (SI Fig. 9).

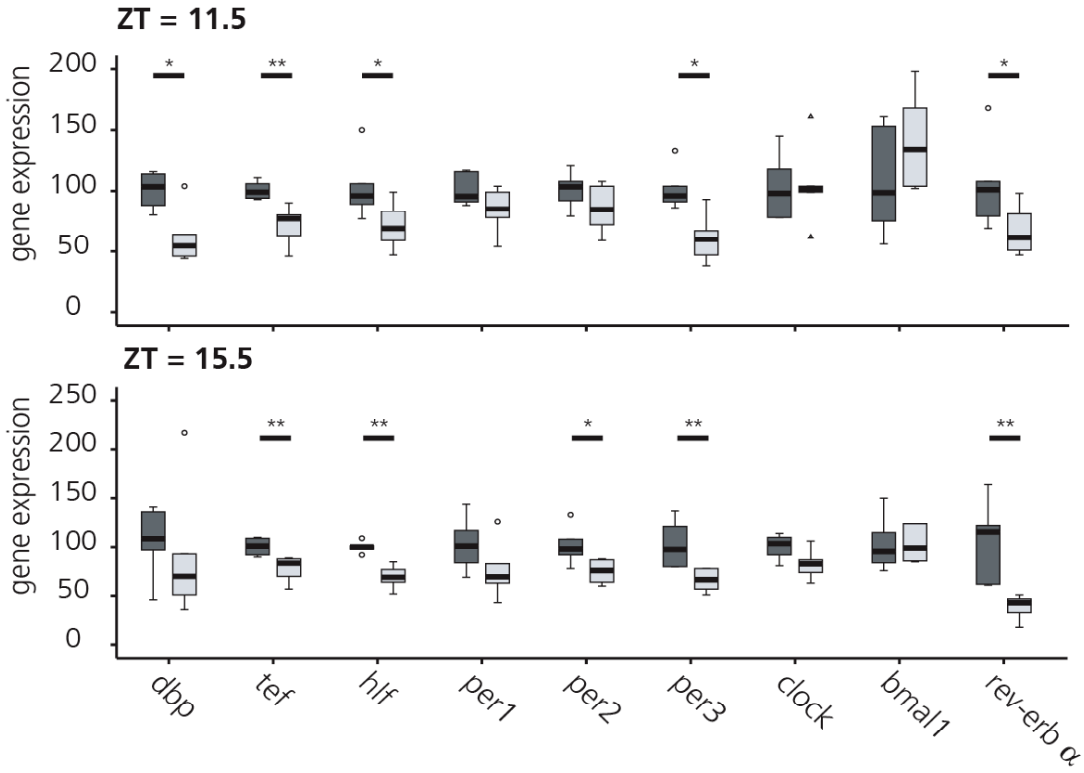

Fig. 4: Impaired expression of clock genes in livers of TNF-α infused mice. Animals were entrained to a LD cycle during 2 weeks, and their locomotor activity was constantly monitored via a passive infrared sensor and a running-wheel. Mice were then implanted with osmotic minipumps delivering TNF-α (light gray bars) or saline as control (dark gray bars). On the third day after the operation, mice were killed at ZT = 11.5 or = 15.5 (ZT = 0, light on; ZT = 12, light off). Livers were extracted and gene expression assessed by real-time PCR. (A) *Per1/2/3*, *Rev-Erbα* and PAR bZip family genes, *Dbp, Hlf*, and *Tef*, are down-regulated at both time points, the suppression of PAR bZip family genes is highly significant. In contrast to fibroblast data, *Rev-Erbα* is strongly down-regulated. Results are shown as percentages relative to the mean of the control group. One representative experiment of three is shown (n = 6 per group; Mann-Whitney test. *, $P \leq 0{,}05$; **, $P \leq 0{,}005$).

TNF-α reduces *Dbp* expression in the SCN in the hypothalamic region.

To verify whether the effect of TNF-α is sustained across the blood-brain barrier and reaches the SCN, the expression of *Dbp* was quantified. As described above, mice were implanted with minipumps releasing TNF-α or diluent; mice were killed after 3 days at ZT 6, when peak expression of *Dbp* was expected. A slight (-15%), but statistically significant reduction of *Dbp* expression ($P = 0.003$) can be seen by *in situ* hybridization in the SCN of TNF-α-treated mice (SI Fig. 10). To verify the time point of the rhythm, we used *Bmal1*, which, at peak expression of *Dbp*, is not detectable. Indeed, unlike *Dbp* mRNA-positive cells, *Bmal1* transcripts were not identified by *in situ* hybridization at ZT 6.

TNF-α reduces locomotor activity and promotes increased rest time in mice

The function of TNF-α to interfere with the transcription of distinct clock genes or CCGs prompted us to evaluate whether TNF-α alters the circadian rhythm and/or the amount of locomotor activity *in vivo*. The timing and extent of locomotor activity were assessed with infrared sensors as well as by monitoring running-wheel activity in mice recorded under a 12-h light/12-h dark (LD) schedule. The surgical procedure led to a decrease in locomotor activity that lasted for 48 h (infrared; Fig. 5A Upper). However, beside the more pronounced impairment of locomotor activity on days 1 and 2, TNF-α led also to a severe suppression of locomotor activity on day 3; on this day, control mice had attained baseline levels of activity. When running-wheel activity was assessed, an analogous picture emerged with maximum inhibition at day 3 (Fig. 5 A Lower and B). At later time points, especially on days 5 and 6, TNF-α no longer exerted any effect on locomotor activity. This is most likely due to a failed release of TNF-α from the pump, because starting at day 4, hemorrhagic necrosis developed at the site of the pump. This local side effect was noticed when pumps were filled with TNF-α but not with control solution and has been described in areas of s.c. injection of TNF-α in mice (3 μg for 5 days) (29). Our results show that TNF-α leads to a decrease in the total amount of locomotor activity as measured by a

running-wheel and infrared sensors until day 4 (Fig. 5A). The activity in both groups was still restricted to the dark period, and highest loss of activity in TNF-α treated animals can be observed during the second half of the active period (Fig. 5B). Further, based on these observations, we chose day 3 to investigate the frequency and duration of rest episodes during the LD period (SI Fig. 11A). During the light period, no change was observed. But in the dark period, when mice are usually active, a significant increase in rest episodes lasting 6-60 min was detected. As readout of the endogenous circadian clock, we assessed locomotor activity in constant darkness ("free-running condition") after implantation of the minipumps and calculated the period length (τ). We observed no changes in the periodicity of τ (SI Fig. 11B). Taken together, mice treated continuously with TNF-α show reduced motor activity and more consolidated rest time but no change in period length under free-running conditions. These findings are consistent with experiments with fibroblasts showing that the amplitude of the expression of clock genes and CCGs are attenuated by TNF-α, rather than their circadian rhythm itself.

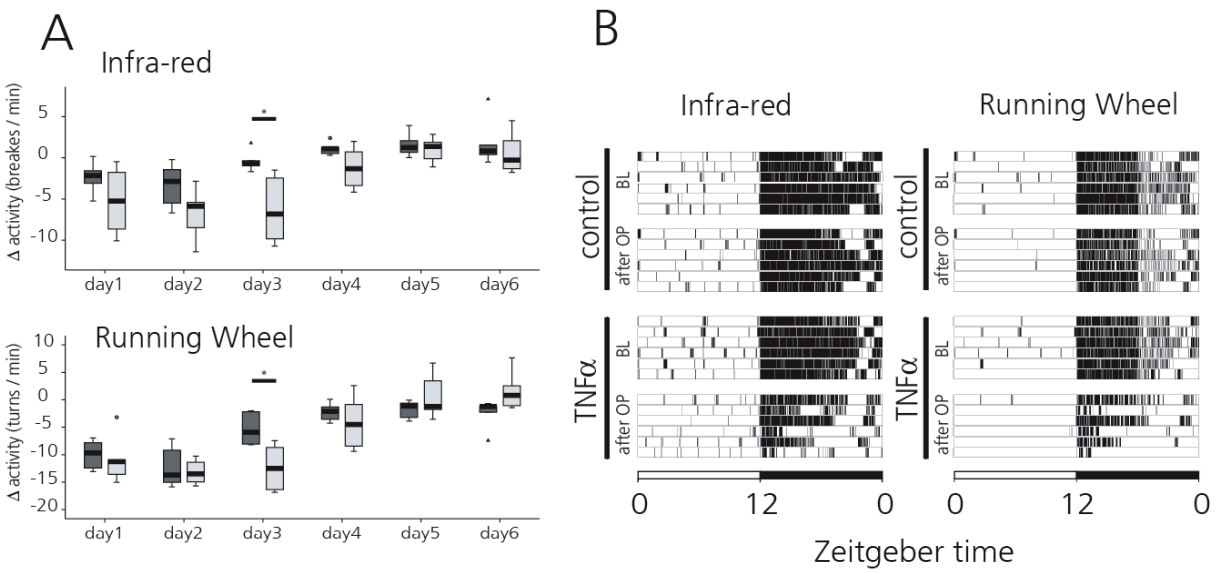

Fig. 5: TNF-α reduces locomotor activity of mice but does not alter their circadian rest-activity cycles. (A) Reduced locomotor activities in mice with constant TNF-α infusions are detected from day 1-4. Boxplot showing the changes of locomotor activity for each day after minipump implantation (TNF-α, 1.5 μg/day, for 6 days) or saline as control, compared with the baseline (mean of 3 days immediately before the operation). The TNF-α-treated group is shown as light gray bars and the control group as dark gray bars; circles are outliers, and triangles are extreme values. Six mice per group were analyzed. Data show one experiment (ANOVA for repeated measures, followed by independent-sample *t*-tests; *, $P \leq 0{,}05$). The effect of TNF-α to impair locomotor activity was confirmed at day 3 in two independent experiments. (B) TNF-α reduces locomotor activity in the second half of the dark phase. Actograms (as measured by infrared and running-wheel) show data from six individual mice before and after the minipump insertion. Each line represents one individual mouse per treatment, 1 day before the implantation of minipumps (baseline, BL) and 3 days after the operation (OP).

Discussion

Whereas the molecular mechanisms provided by clock genes to maintain the circadian rhythm are becoming increasingly clear, the potential influences of the immune system on the molecular clockwork remain to be explored. Here, we provide evidence that TNF-α interferes with the expression of clock genes, namely the *Per* genes and the PAR bZip genes *Dbp*, *Tef*, and *Hlf*. TNF-α suppresses the expression of these genes in fibroblasts (and attenuates their amplitudes) *in vitro* and *in vivo* in the liver of mice infused with this cytokine. That the same genes (with the exception of *Rev-Erbα*) are prone to TNF-α stimulation *in vitro* compared with *in vivo* speaks for a rather direct effect of TNF-α, although we cannot exclude other intermediates

involved leading to the same response. *In situ* hybridization shows that the s.c. administration of TNF-α also leads to reduction of *Dbp* expression in the central circadian pacemaker, the SCN. Thus, it is tempting to speculate that TNF-α is likely to reach the SCN via the blood and to bind to TNF receptor (TNF-R) on neurons. At least in the hippocampus, both TNF-RI and -RII, are expressed (30). In the present study E-box regulatory elements of clock genes are found to play a pivotal role in the effect of TNF-α to inhibit clock gene expression. First, only clock genes with E-boxes in their promoter, the PAR bZip genes *Dbp*, *Tef*, and *Hlf* and the *Per* genes, are affected by TNF-α, whereas clock genes devoid of E-boxes such as *Clock* and *Bmal1* are not affected by TNF-α. Second, mutated E-boxes provide protection of TNF-α-induced suppression of clock reporter genes. E-boxes are functionally important components of DNA promoters that guide the expression of clock genes and thereby influence the circadian rhythm, including the sleep-wake cycle. Rhythmic binding of CLOCK and BMAL1 depends on E-boxes and is a prerequisite for robust waves of gene expression characteristic of circadian transcription (31, 32).

The effects of TNF-α on clock gene expression also become apparent when studying clock-dependent genes. *Dbp* has been described to mediate transcription of the albumin gene in hepatocytes (25). Albumin serum concentrations are found here to be lowered by 41% in TNF-α-treated mice compared with controls.

Recording of locomotor activity of TNF-α-treated mice shows more rest episodes during spontaneous activity. In line with the finding that TNF-α does not alter circadian rhythm in cultured fibroblasts but rather lowers the extent of expression of distinct clock genes, TNF-α did not influence period length of the circadian rhythm under "free running" conditions. Of interest for the findings presented here are data showing that the deletion of the *Dbp* gene in mice results in only a slight reduction of period length but in a striking impairment of spontaneous locomotor activity and running-wheel activity (33, 34). Whereas *Tef* and *Hlf* single-knockout mice show an increased period length, the inactivation of all three genes, *Dbp*, *Tef*, and *Hlf*, resulted in an unchanged circadian period length (27). Thus, a normal period length may be due to opposite effects leading to mutual neutralization of indirect clock gene dysfunction. Besides lowering the expression of PAR bZip transcription factors, TNF-α

impaired *Per1, Per2*, and *Per3* mRNA. Target disruption of these genes results in slightly (*Per3*$^{/-}$ mice) or more dramatically (*Per1*$^{/-}$ mice) shortened period lengths or eventually gives rise to arrhythmic behavior (*Per2*$^{/-}$ mice). Taken collectively, TNF-α interferes with the expression of E-box-dependent clock genes and leads to prolonged rest episodes during spontaneous activity of mice.

Sleep architecture in humans is affected by the endogenous circadian pacemaker, which regulates the timing of the sleep-wake cycle, presumably by circadian expression of clock genes (13, 35). Enhanced NREM sleep has been shown in mice treated with muramyl dipeptides, which activate TLR2 and TLR4 on macrophages (36, 37). TNF-α, as well as IL-1, is produced by TLR2- and TLR4-activated macrophages and is well described to enhance NREM sleep (11, 38, 39). In light of the overlapping properties of TNF-α and IL-1 on NREM sleep, it is interesting that our studies show IL-1 to share with TNF-α the inhibitory effect on expression of the *Dbp* and *Per3* genes in fibroblasts. As recently outlined, the effects of IL-6 on sleep differs from IL-1 and TNF-α, in that it may also act on systems involved in NREM sleep but not in REM sleep, which is suppressed by TNF-α and IL-1 but not by IL-6 (40). In this context, it may be of relevance that the expression of *Dbp* is found here not to be affected by IL-6. Although the expression of *Dbp* and *Per1* is also not inhibited by IFN-α, IFN-γ only slightly interfered with *Dbp* expression. This is remarkable, because in mice, the daily injection of IFN-α or -γ has been reported to lower P*er1, Per2, Per3*. and *Clock* after 6 days of treatment (41). The absence of effects of IFN-α and -γ on expression of the *Per* genes by synchronized fibroblasts *in vitro* indicates that IFNs down-regulate the *Per* and *Clock* genes by E-box-independent mechanisms or induce production of other clock gene-regulating factors when mice are treated over a long time period with the cytokines.

In infectious diseases, TNF-α serves to successfully eliminate the infectious agent. The function of TNF-α to interfere with the expression of clock genes, to impair locomotor activity, and to enhance rest may provide the link between the activation of the innate immunity and fatigue associated with infectious and autoimmune diseases, such as multiple sclerosis, RA, or Crohn's disease. In these disorders, both fatigue and elevated TNF-α gene concentrations have been described (6, 42, 43). It

is still debated whether sleep changes in infections are beneficial to the host defense. Rabbits infected with *E. coli*, *Staphylococcus aureus*, or *Candida albicans* showed an improved prognosis when their sleep duration was prolonged (44). In this context, it is to be noted that *Per2*$^{-/-}$ mice are partially protected from LPS-induced shock (45). During the TNF-α induced "inflammatory clock gene response", the expression of *Per1*, *Per2*, and *Per3* genes and of the PAR bZip transcription factors *Dbp*, *Tef*, and *Hlf* is down-regulated, the locomotor activity reduced, and rest episodes prolonged. Although this pathway may induce an adaptive state in infectious diseases, the "inflammatory clock gene response" may, by inducing fatigue, diminish quality of life in autoimmune diseases. Our study will serve to lay an important foundation for further exploration of the connection between the TNF-α-induced "inflammatory clock gene response" and the TNF-α-triggered reduction of locomotor activity.

Materials and Methods

Cytokines

Recombinant murine (rm) TNF-α, rm IL-1β, and rm IL-6 were purchased either from Sigma (St. Louis, MO) (*in vitro* time course assays) or from Peprotech (London, U.K.) (*in vivo* assays); rm IFN-α from Immunotools (Friesoythe, Germany), and rm IFN-γ from Roche (Rotkreuz, Switzerland).

Synchronization of fibroblasts by serum shock

NIH 3T3 fibroblasts were grown in DMEM (Gibco, Basel, Switzerland) supplemented with 10% FBS (PAA Laboratories, Pasching, Austria) and Glutamax (Gibco). For serum shock, cells were grown to confluency in 6-cm tissue culture dishes. At time t = 0, the medium was exchanged by 50% horse serum (Gibco) in DMEM/Glutamax; after 2 h, the medium was replaced with serum-free DMEM/Glutamax, with or without TNF-α. At the indicated time points, tissue culture dishes were washed once with Hanks solution, frozen on a layer of liquid nitrogen, and kept at –70°C until the extraction of whole-cell RNA.

Transfection and luciferase assays

Unsynchronized NIH 3T3 cells were transfected with the following constructs by using Lipofectamine Plus (Life Technologies, Basel, Switzerland) or Trans-Fectin (BioRad, Hercules, CA) according to the manufacturer's protocol: mPer1-luc (46), kindly provided by David Earnest (Texas A&M, College Station, TX); E54-TK (22), kindly provided by Sato Honma (Hokkaido University, Sapporo, Japan); Bmal1-luc (19), DBP-Ebox-luc and mut Ebox, pCDNA3.1-Clock and pCDNA3.1-Bmal1 (47), kindly provided by U. Schibler (University of Geneva, Geneva, Switzerland); mPer3-luc (48), kindly provided by P. Sassone-Corsi (IGBMC, Illkirch, France). As an internal control for transfection efficiency, a GFP construct (pMax-GFP; Amaxa, Cologne, Germany) was cotransfected 1:10.

Twenty-four hours after transfection, the medium was replaced with serum-free DMEM/glutamax with or without TNF-α (10 ng/ml). After $\approx$15 h cells were lysed by using Passive Lysis Buffer (Promega, Wallisellen, Switzerland) and enzyme activity was measured by the Luciferase Assay System (Promega). Bioluminescence was measured with a Luminometer (Berthold Technologies, Regensdorf, Switzerland) and normalized to transfection efficiency or protein concentration.

Animal groups and locomotor activity recording

Seven-week-old C57Bl/6 male mice (Harlan Breeding Laboratories, AD Horst, The Netherlands) were housed in individual cages, equipped with a running-wheel and a passive infrared sensor in a temperature-controlled sound-proof light-tight room. Food and water were available *ad libitum*. We allowed mice 10-15 days of acclimatization to a LD cycle (light on at 0600, i.e., ZT 0; light off at 1800, i.e., ZT 12). Mice were operated under deep isoflurane anaesthesia, and 30 μg Temgesic anesthetic (buprenorphine; Essex Chemie, Lucerne, Switzerland) was applied. TNF-α (1.5 μg/day, diluted in 0.1% BSA/PBS) or 0.1%BSA/PBS as a control was administered s.c. by using osmotic minipumps (Model 1007D; Alzet, Cupertino, CA) implanted on the back, for 6 days. Locomotor activity was continuously measured via running-wheel and infrared sensors based on 1-min episodes by using the Chronobiology Kit software (Stanford Software Systems, Santa Cruz, CA) as

described (49, 50). Rest episodes were defined as 1-min units with activity = zero. The free-running period of locomotion was calculated by periodogram analysis for days 2-6 after minipump implantation, when the mice were kept in constant darkness. In gene expression studies, livers were extracted 3 days after minipump insertion at two different ZTs known to approximately represent the peak of expression of *Dbp* (ZT = 11.5) or *Per2* (ZT = 15.5). Livers were frozen in TRIzol (Invitrogen, Basel, Switzerland) for subsequent RNA extraction. All experimental procedures were approved by the local committee of the veterinary office and in strict accordance with Swiss regulations on animal welfare.

RNA isolation and gene expression analysis

The method for RNA extraction, RT-PCR, and quantification of gene expression is described in the *SI Text*.

In situ hybridization

The method for *in situ* hybridization was published (51) and is described in the *SI Text*.

Acknowledgments

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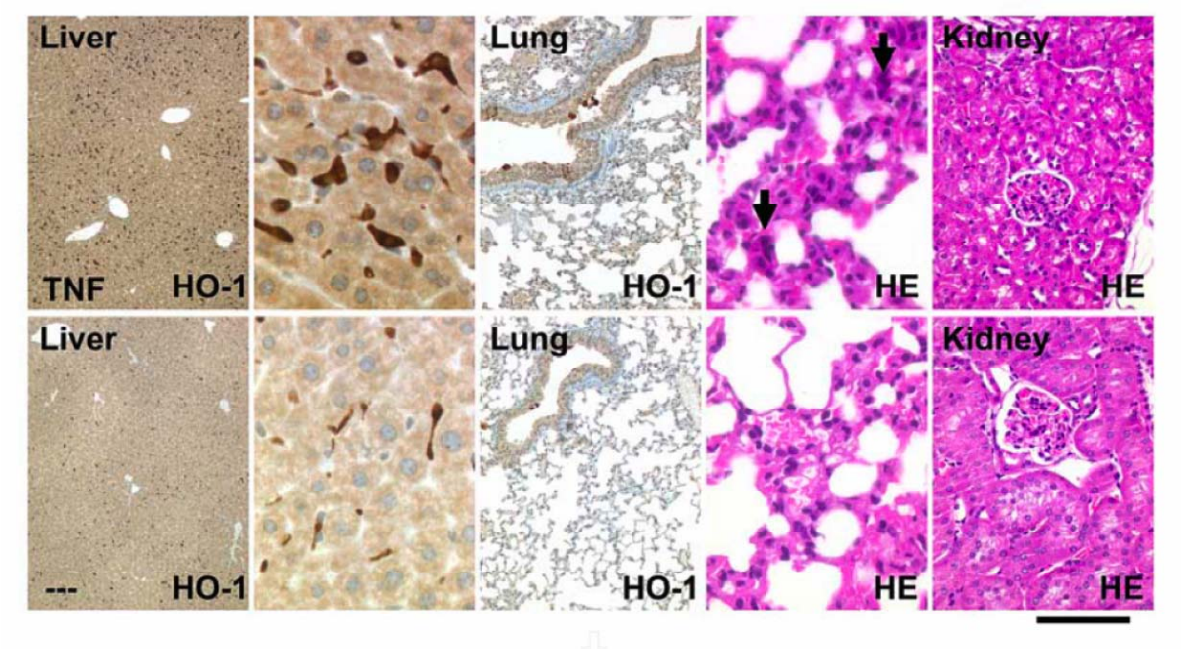
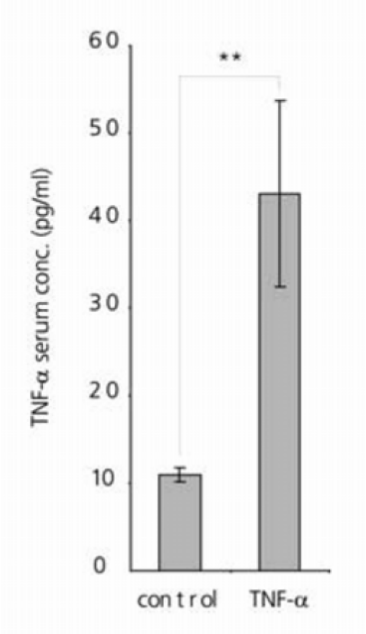
Suporting information

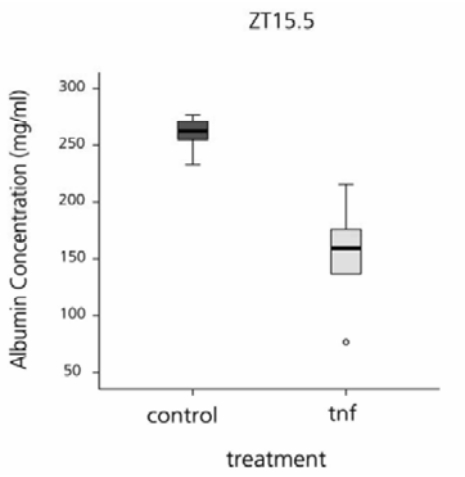
SI Figures



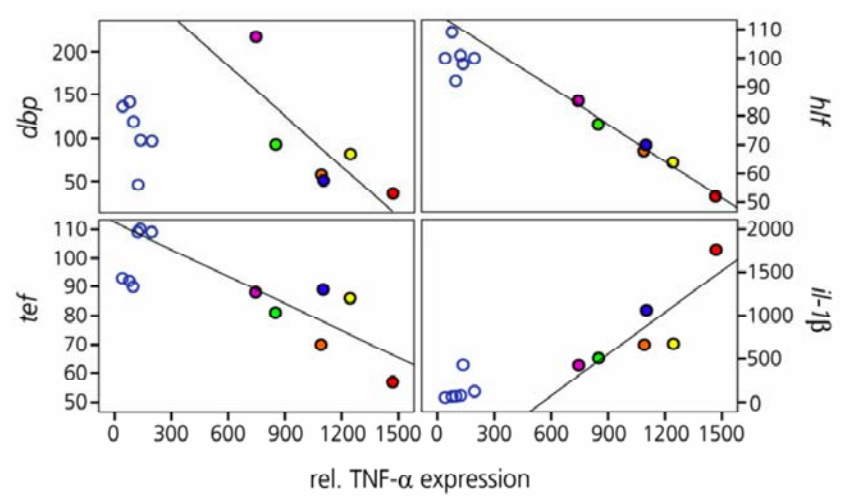
SI Fig. 6. Photomicrographs of histological sections of liver, lung and kidney with (top row) and without (bottom row) TNF-α treatment: The liver shows an increase in HO-1 positive Kupffer cells. At high power magnification the cells appear also larger. No morphologically apparent liver cell damage is however detected. The lung shows unremarkable numbers of HO-1 positive macrophages in the bronchial mucus layer. No increase in HO-1 positive alveolar macrophages is seen. At high power magnification (hematoxillin eosin staining) increased numbers of megakaryocytes (indicated by arrows) are seen trapped within the alveolar vascular bed. There is, however, no evidence of pulmonary edema or acute pulmonary damage. Sections of the kidney (hematoxillin eosin staining) are unremarkable. The scale bar represents from left to right 400 μm, 50 μm, 200 μm, 50 μm and 100μm, respectively.



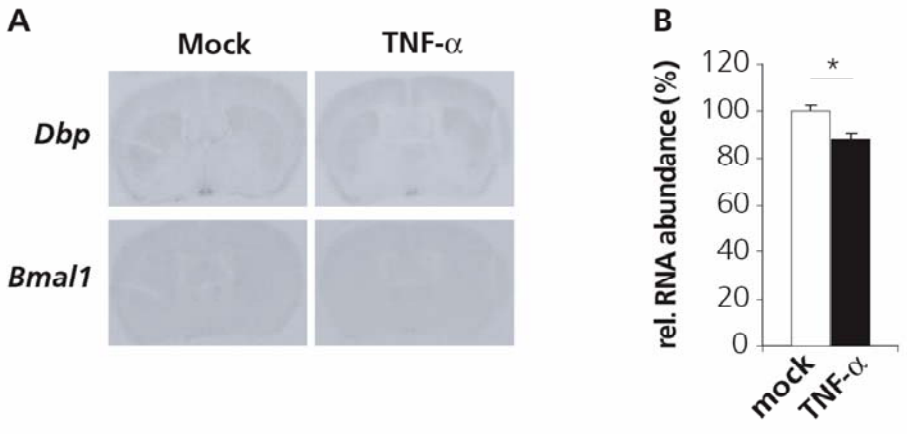
SI Fig. 7. At day 3 (ZT 15.5) after minipump insertion, serum was collected and TNF-α was measured by ELISA. Data are given as the mean ± SD of TNF-α serum concentrations measured in six animals per group. Independent samples *t*-test, $P = 0.001$.



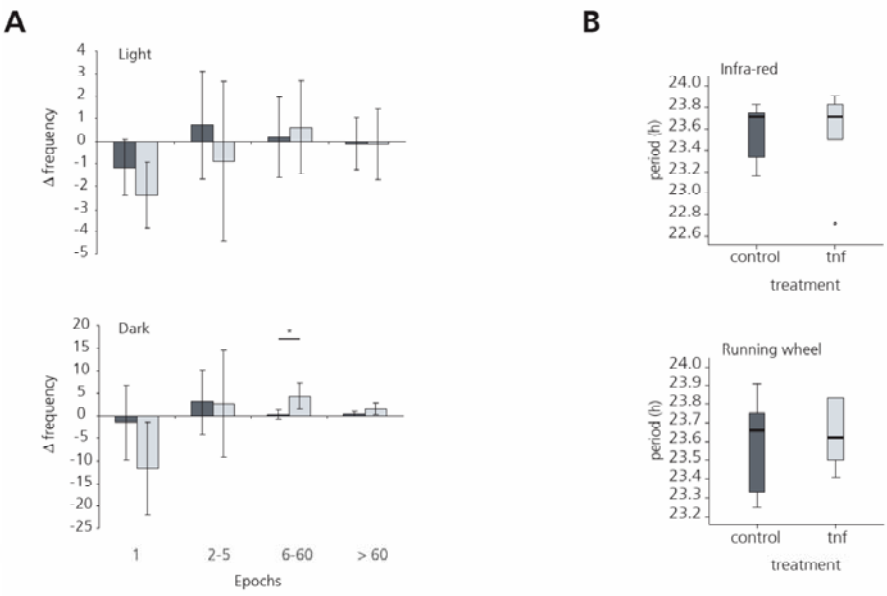
SI Fig. 8. Decreased concentrations of albumin in serum of TNF-α-treated mice. Serum was collected at day three after minipump implantation at ZT 15.5 and albumin concentrations were measured as described in materials and methods. Data show the mean ± SD of serum albumin concentrations in mice (six mice per group).



SI Fig. 9. IL-1β expression correlates to TNF-α expression, both being increased in TNF-α-treated animals (circles with different colors, each individual mouse representing one color) compared to animals infused with PBS (blue open circles) (day 3, at ZT 15.5; 6 mice per group). In contrary, *Dbp*, *Hlf*, and *Tef* are negatively correlated to the relative TNF-α expression.



SI Fig. 10. TNF-α impairs expression of the clock output gene *Dbp* in the SCN. (A and B) *Dbp* and *Bmal1* expression in the SCN at ZT 6 as determined by radioactive *in situ* hybridization. At the time point of peak expression of *Dbp,* when *Bmal1* expression is lowest, TNF-α leads to a significant reduction by 15% of the *Dbp* mRNA. (means ± SEM of six consecutive sections per mouse SCN; four mice; paired *t*-test; * *P*=0.003).



SI Fig. 11. (A) Mice treated with TNF-α show increased long rest epochs in the dark phase. Analysis of rest epochs was performed at day 3 of TNF-α and control infused mice; the analysis being performed separately for the light and dark period. The data show the differences in numbers of 1-min episodes with activity = 0 compared to baseline (mean of the three days before minipump insertion). The occurrence of rest was arbitrarily subdivided in episodes for durations with rest = 0 activity counts: up to 1 min rest, between 2 and 5 min, 6 and 60 min, and more than 60 min. (ANOVA for repeated measures, followed by independent-samples *t*-test; * $P \leq 0{,}05$). (B) TNF-α does not affect the period length measured in constant darkness. On day 1 after minipump insertion the lights were turned off and the mice were kept in constant darkness. The circadian period of locomotion and running-wheel activity was determined by periodogram analysis over the 5 days in constant darkness. Independent-sample *t*-tests were performed. TNF-α (1.5 μg/day; light gray bars) or saline as control (dark gray bars).

Gene name	forward primer 5' - 3'	probe 5'-FAM - 3' -TAMRA	reverse primer 5' - 3'
dbp	GCGAGAAGTGCAAAATTGGC	CGCGCGCCTGTGTCCCTTG	CGGGAGGCTCCTATAGTCTGG
hlf	CGCCAGGAGGTGGCTG	TTTAAGGAAGGAGCTGGGCAAATGCAA	GCCTCGTACTTGGCAAGTATGTT
rev-erb a	ACAGCAGCCGAGTGTCCC	CAGCAAGGGCACAAGCAACATTACCAAG	ACACAGTAGCACCATGCCATTC
bmal1	CCAAGAAAGTATGGACACAGACAAA	TGACCCTCATGGAAGGTTAGAATATGCAGAA	GCATTCTTGATCCTTCCTTGGT
clock	TTGCTCCACGGGAATCCTT	ACACAGCTCATCCTCTCTGCTGCCTTTC	GGAGGGAAAGTGCTCTGTTGTAG

	pre-developed taqman assays (Applied Biosystems)
18s rRNA	4310893E
Per1	Mm00501813_m1
Per2	Mm00478113_m1
Per3	Mm00478120_m1
Tef	Mm00457513_m1

SI Table 1. Sequences for primers and probes, normal concentrations used were 250 nM for Probes and 400nM for the primers.

SI Text

RNA isolation and gene expression analysis

Whole-cell RNA from cultured cells was extracted using the NucleoSpin-RNA II kit (Macherey-Nagel, Switzerland). RNA from mouse tissues was extracted by homogenization of the organ in TRIzol (Invitrogen) according to the manufacturer's instructions. Subsequently, RNA was reverse-transcribed using random hexamers (Promega, for the *in vitro* assays; Roche, for the *in vivo* assays) and AMV reverse transcriptase (Promega, for the *in vitro* assays) or M-MuLV reverse transcriptase (Roche, for the *in vivo* assays). The cDNA equivalent to 50 ng of total RNA was PCR-amplified in an ABI PRISM 7700 detection system (PE-Applied Biosystems) using the TaqMan Universal PCR Master Mix (Applied Biosystems) and quantified as follows. Primers and probes for Taqman analysis were either purchased from Applied Biosystems or purchased from Microsynth, Balgach Switzerland as described in detail in SI table 1. The relative levels of each RNA were calculated by the $2^{-\Delta\Delta CT}$ method (CT standing for the cycle number at which the signal reaches the threshold of detection); *18s rRNA* was used as a housekeeping gene. Each CT value used for these calculations is the mean of two duplicates of the same reaction. Relative RNA levels are expressed as x-fold variations compared to ZT = 0 (time course experiments) or as percentages of the average control groups (in the *in vitro* one-time-point experiments and in the *in vivo* experiments).

In situ hybridization

After infusion of mice with saline or TNF-α mice were killed at day three at ZT 6. Brains were embedded in paraffin and sectioned at 7-µm-thickness and hybridized with 35S-rUTP labeled riboprobes as described (51). The *Bmal1* probe corresponded to nucleotides 654-1290 (accession no. AF015953) and the *Dbp* probe to nucleotides 2-951 (accession no. NM016974). Quantification was performed by densitometric analysis of autoradiograph films using the Molecular analyst program (Bio-Rad). Data from the SCN were normalized to the lateral hypothalamus next to the SCN. For each treatment three animals were used and six sections per SCN were analyzed. We

assessed the relative mRNA abundance values by defining the highest value of each experiment in mock treated animals as 100%.

Elisa

The concentration of albumin and TNF-α in the serum of mice implanted with osmotic minipumps was determined by ELISA (Mouse Albumin ELISA Quantitation Kit, Bethyl Laboratories, TX and mouse TNF-α ELISA kit, KMC3012, Biosource).

4.2. CLOCK GENE MODULATION BY TNF-α DEPENDS ON CALCIUM AND P38 MAP KINASE SIGNALING

Petrzilka S., Taraborrelli C., Cavadini G., Fontana A. and Birchler T.

My contributions to this work: I have contributed to all the figures.

Clock gene modulation by TNF-α depends on calcium and p38 MAP kinase signaling

Petrzilka Saskia*, Taraborrelli Cornelia*, Cavadini Gionata*, Fontana Adriano* and Birchler Thomas*

*Division of Clinical Immunology, University Hospital Zurich, Haeldeliweg 4, CH-8044 Zurich

Abstract

Treatment of fibroblasts for 24 hours with tumor necrosis factor-alpha (TNF-α) suppresses transcription of E-box driven clock genes (*Dbp*, *Tef, Hlf* and *Per1*, *Per2*, *Per3*) by yet unknown molecular mechanisms. The attenuation of clock genes has been suggested as a putative cause for the development of sickness behavior syndrome in infections and autoimmune diseases. Here, we studied the effect of TNF-α at early time points (<3 h) on intracellular signaling events and clock gene expression in fibroblasts. Interaction of TNF-α with TNFR1 (Tnfrsf1a, CD120a, p55) leads to downregulation of gene expression of D-site albumin binding protein (*Dbp*) and upregulation of negative regulators of the molecular clock, *Period-1* and *-2* (*Per1* and *-2*), *Cryptochrome-1* (*Cry1*), and *Differentiated embryo chondrocytes-1* (*Dec1*). Since the decrease of *Dbp* is also observed in knockout cells for *Per1/2* and *Dec1*, these genes are unlikely to be responsible for inhibition of *Dbp.* The effect of TNF-α on the clock genes, *Dbp* and *Per1*, in the early phase is dependent on p38 mitogen activated protein kinase (MAPK) and/or calcium signaling but not on the activation of NF-κB or AP-1. Taken collectively the data show p38 MAPK and calcium dependent, TNFR1 mediated transient increase of negative regulators of the molecular clock and an independent decrease of *Dbp*.

Introduction

TNF-α is a pleiotropic cytokine, which regulates the activation and function of a variety of cells including lymphocytes, fibroblasts and neurons (1). Its receptors, especially TNFR1, are ubiquitously expressed (2). In response to infections or in inflammation during autoimmune diseases (e.g. rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease) TNF-α is produced mainly by dendritic cells and macrophages (1). While infusions of TNF-α in cancer patients lead to severe daytime fatigue (3), treatment of patients with rheumatoid arthritis or

obstructive sleep apnoe syndrome with TNF-α blockers reduces disease-associated fatigue (4, 5). On the other hand, a disruption of the circadian clock can lead to the same symptoms. These data indicate a possible influence of TNF-α on the circadian system and thereby on fatigue.

Recently, the effect of TNF-α on the molecular clock was investigated by our group. The data showed that TNF-α reduces the expression of E-box driven clock genes in cultured fibroblasts. Moreover TNF-α impairs nocturnal locomotor activity in mice, the effect being associated with a decrease of clock genes in the liver (6). These results suggest that TNF-α, by interfering with clock gene expression, might attenuate the circadian output of physiological and behavioural rhythms. Here, we aimed to elucidate signaling events leading to reduced clock gene expression, in TNF-α treated fibroblasts.

The molecular clock is the cellular basis of circadian rhythms found in almost every organism ranging from bacteria, plants, and flies to humans. Circadian rhythms control many aspects of physiology and metabolism that allow organisms to anticipate regular changes in the environment (for reviews see (7)). In mammals, the integration of the day and night variation is provided by the suprachiasmatic nucleus (SCN) of the hypothalamus, which receives light information from the retina and synchronizes the clock of other brain areas and peripheral organs. Rhythms in single cells are thought to origin from interlocked transcription-translation feedback loops composed of positive transcriptional activators *Clock* and *Bmal1* and negative elements such as *Per* and *Cry*. CLOCK and BMAL1 heterodimerize and bind to E-box enhancer sequences to promote transcription of *Per1*, *Per2*, *Cry1*, and *Cry2*, the products of which inhibit the activity of CLOCK:BMAL1 and hence their own transcription. A second feedback loop controls the expression of the positive clock element: REV-ERBα - itself activated through E-boxes by CLOCK:BMAL1 - rhythmically inhibits *Bmal1* transcription (8). Post-translational modifications, such as phosphorylations, acetylations, sumoylations, among others allow for the fine tuning of the system generating a period of about 24 hours. Clock-controlled genes (CCGs) such as *Per3* and the members of the PAR-bZip family of transcription factors, *Dbp*, *Tef*, and *Hlf*, also bear E-box motifs and their periodical expression contributes to the transformation of time information into physiological and behavioural rhythms. However, they are not integral part of the timing system.

TNF-α signals via two receptors: TNFR1 (p55TNFR, CD120a, TNFRSF1a) and TNFR2 (p75TNFR, CD120b, TNFRSF1b). Whereas TNFR1 is ubiquitously expressed and more effective in sensing soluble TNF-α; TNFR2 has a higher affinity to the membrane bound cytokine, is involved in ligand passing to TNFR1 and bears its main functions in chronic inflammatory conditions. After receptor activation different intracellular adaptor proteins direct a complex array of signaling processes mediated by kinases, phosphatases, reactive oxygen intermediates, lipases, caspases, and sphingomyelinases. This leads to regulation of the cell function by transcription factors, G-protein signaling, and calcium ion signaling. In the TNF-α dependent intracellular signaling cascade, activation of caspases and the transcription factors NF-κB and AP-1 are important steps and have been found to regulate apoptosis and expression of chemokines and cytokines.

This study was aimed to characterize the relevant intermediates of TNF-α signaling leading to altered clock gene expression. In the early response of fibroblasts to TNF-α, transcription of negative clock gene regulators, namely *Per1/2*, *Cry* and *Dec1* is activated. However, this induction is unlikely to be involved in downregulation of the clock-controlled gene *Dbp.* Impaired expression of *Dbp* was not dependent on caspase signaling, NF-κB, or AP-1 activation, but rather on the calcium ion signaling. Although there is less binding of acetylated histone H3 to the *Dbp* E-box 857, active deacetylation is not involved in the inhibition of *Dbp* by TNF-α.

Results

Immediate early induction of negative clock elements parallels fast *Dbp* downregulation.

To elucidate the direct effects of TNF-α on clock gene expression, we followed the immediate early response in NIH 3T3 fibroblasts. Upon treatment with TNF-α, expression of the clock controlled gene *Dbp* started to decrease after 15 min and reached 20% of control after 2 h (Fig. 1A, top left). A more delayed downregulation was observed when analyzing the expression of *Per3* (Fig. 1A bottom middle). In

contrast, *Per1* - and to a lower extent also *Per2* - showed a significant transient peak of expression at 1 h after stimulation (Fig. 1A and B), with subsequent downregulation at later time points. Whereas both *Cry1* and *Dec1* were successively upregulated, the effect of TNF-α on *Cry2* and *Dec2* was less pronounced and the expression of *Clock* was hardly affected (Fig. 1A). The *Per1* gene is regulated by two alternative promoters (13) regulating two alternative first exons, i.e. 1A and 1B. There was no difference in the *Per1* peak induction at 1 h at the different promoters indicating similar regulation of both promoters (Fig. 1C). Taken collectively the data show that the clock genes analyzed respond differently to TNF-α treatment in a time dependent manner.

In further experiments we tested if TNF-α induces increased degradation of *Dbp* mRNA. When blocking RNA transcription with Actinomycin D, no enhanced degradation of the short-lived *Dbp* mRNA was observed (Fig. S1A). However, we still found downregulation of pre-mRNA transcription by analysis of nascent transcripts (Fig. S1B). Hence, the effect of TNF-α to downregulate *Dbp* mRNA is due to a block in transcription and to the short lifespan of the mRNA.

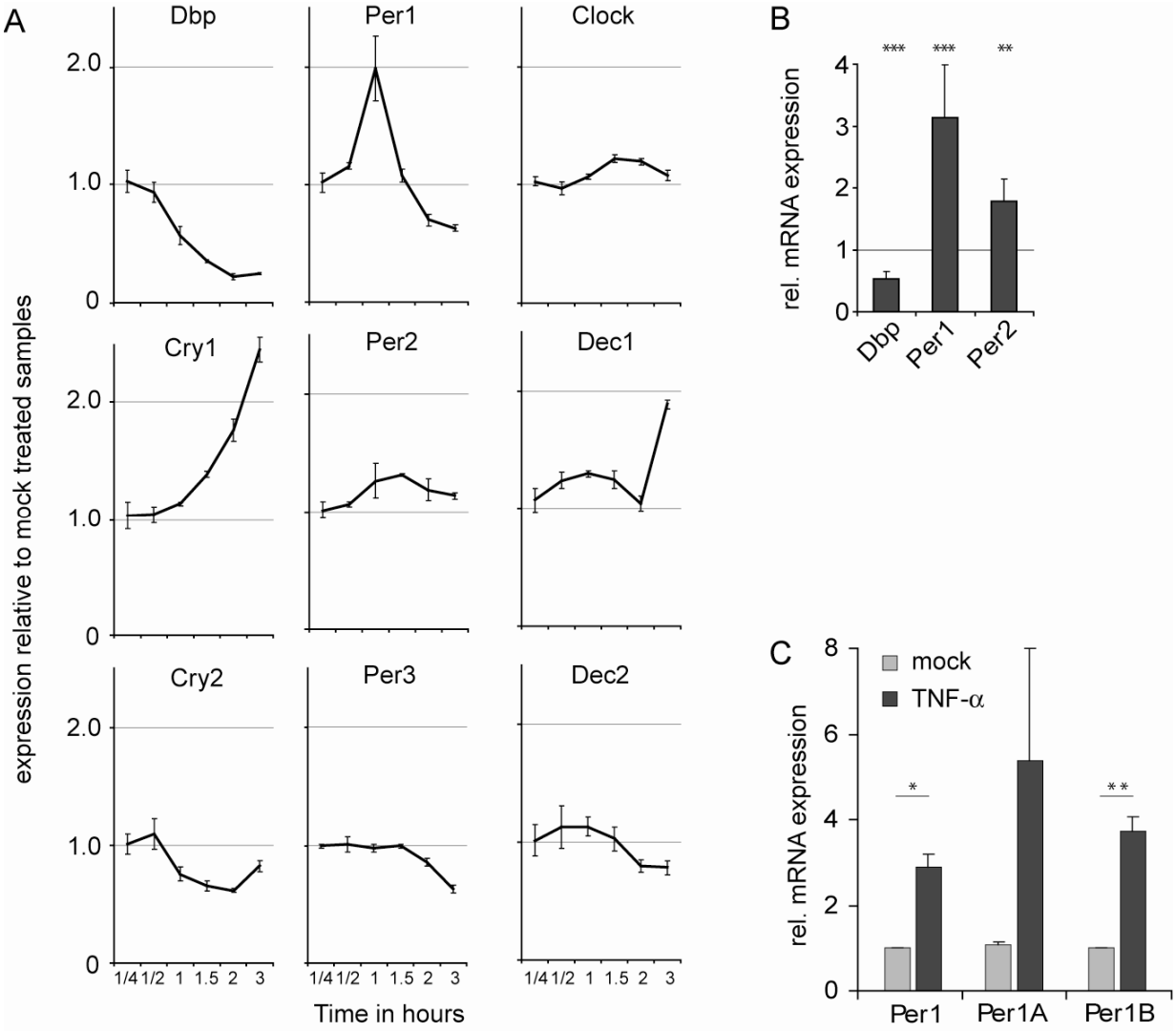


Fig. 1: Early expression time course of clock genes in TNF-α treated NIH 3T3 fibroblasts. (A) Cells were stimulated with 10 ng/ml TNF-α in serum-free medium and mRNA expression of clock genes was analyzed at the indicated time points. Shown is the mean ± SD from 1 experiment in triplicates out of 2. (B) *Per1* and *Per2* are significantly induced and *Dbp* significantly inhibited after 1 h stimulation with TNF-α. Shown is the mean + SD; n=10 for *Per1* and *Dbp*, n=6 for *Per2*. (C) Induction of *Per1* is mediated by promoter 1A and 1B. NIH 3T3 were stimulated with 10 ng/ml TNF-α for 1 h; n=3 in triplicates. Shown is the mean + SD. For B and C we used paired sample t-tests; *, p≤ 0.05; **, p ≤ 0.005; ***, p ≤h 0.0005

Cycloheximide partly de-represses *Dbp* inhibition

To assess the dependency of *Dbp* downregulation on *de-novo* induced proteins, we performed assays with cycloheximide (CHX), a compound inhibiting protein biosynthesis. Five hours before TNF-α treatment, CHX was added to the cell cultures to inhibit protein translation. Thereafter, TNF-α was added only for 2 h since a longer exposure was found to induce apoptosis. The inhibition of *Dbp* expression was reversed but could not be de-repressed completely (Fig. 2). These results show that

the downregulation of *Dbp* is partly dependent on *de-novo* protein synthesis e.g. of a short-lived protein, but cannot be fully explained by a newly produced putative inhibitor, a fact also supported by the fast kinetics of *Dbp* downregulation.

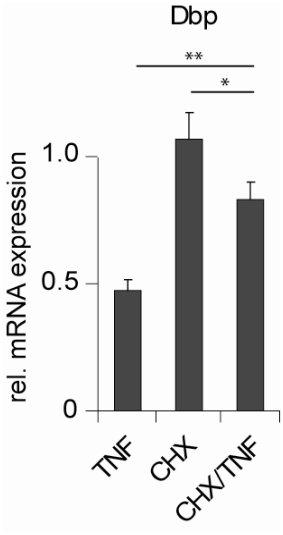


Fig. 2: The inhibition of *Dbp* is partly dependent on *de-novo* protein synthesis. After a 5 h pretreatment with cycloheximide (100 µg/ml) NIH 3T3 fibroblasts were stimulated with TNF-α for 1 h. Expression of untreated samples corresponds to 1. Shown is the mean + SD; n=3 in triplicates; paired samples t-test; *, $p \leq 0.05$; **, $p \leq 0.005$.

The transient induction of negative regulators by TNF-α is not involved in later *Dbp* repression

Mammalian *Per* genes are necessary for proper circadian rhythm generation. The induction of *Per1* which acts as a negative feedback regulator could be important for the long-lasting TNF-α mediated downregulation of clock genes. Increased *Per* gene expression could lead to increased PER levels with putatively enhanced stability and activity by posttranscriptional modifications. To evaluate this hypothesis, we analyzed mouse embryonic fibroblasts (MEFs) lacking *Per1*, *Per2*, or both of them (11); those were compared to MEFs from corresponding wt mice. The baseline expression of the clock genes, *Dbp*, *Clock*, and *Bmal1* is strongly reduced in *Per1* -/- and lower in *Per2* -/- MEFs. For unknown reasons the expression of *Dbp* in *Per1/2* -/- was not different compared to wt MEFs (Fig. 3A, left panel). However, in regard to the TNF-α response it becomes clear that the ability of TNF-α to downregulate *Dbp* gene expression was

still present in all genotypes when compared to baseline levels (Fig. 3A, right panel). Only a moderate loss of effect of TNF-α was seen in *Per1 -/-* and *Per2 -/-* MEFs cells, which however was not seen in double mutant cells. A faint upregulation of *Clock* and *Bmal1* was observed in all genotypes upon TNF-α challenge (Fig. 3A right panel). In conclusion, the mutations of *Per* genes had no major impact on the effects of TNF-α on the clock genes analyzed.

We also analyzed whether the TNF-α induced increase of *Dec1* (Stra13/Sharp2/BHLHB2) is responsible for downgegulation of *Dbp* (Fig 1A). This possibility raised because of recent data on DEC1 to impair E-box mediated transcription (14). Our data show that TNF-α effectively repressed *Dbp* gene transcription also in the human keratinocyte cell line, HaCaT. However, this effect was not de-repressed in *Dec1*-deficent HaCaT cells which have a targeted inactivation of *Dec1* on both alleles through homologous recombination (Fig. 3B) (15). Taken collectively, our data show that despite of being upregulated at early time points neither PER1 and PER2 nor DEC1 seem to act as *Dbp* repressor molecules in TNF-α treated cells.

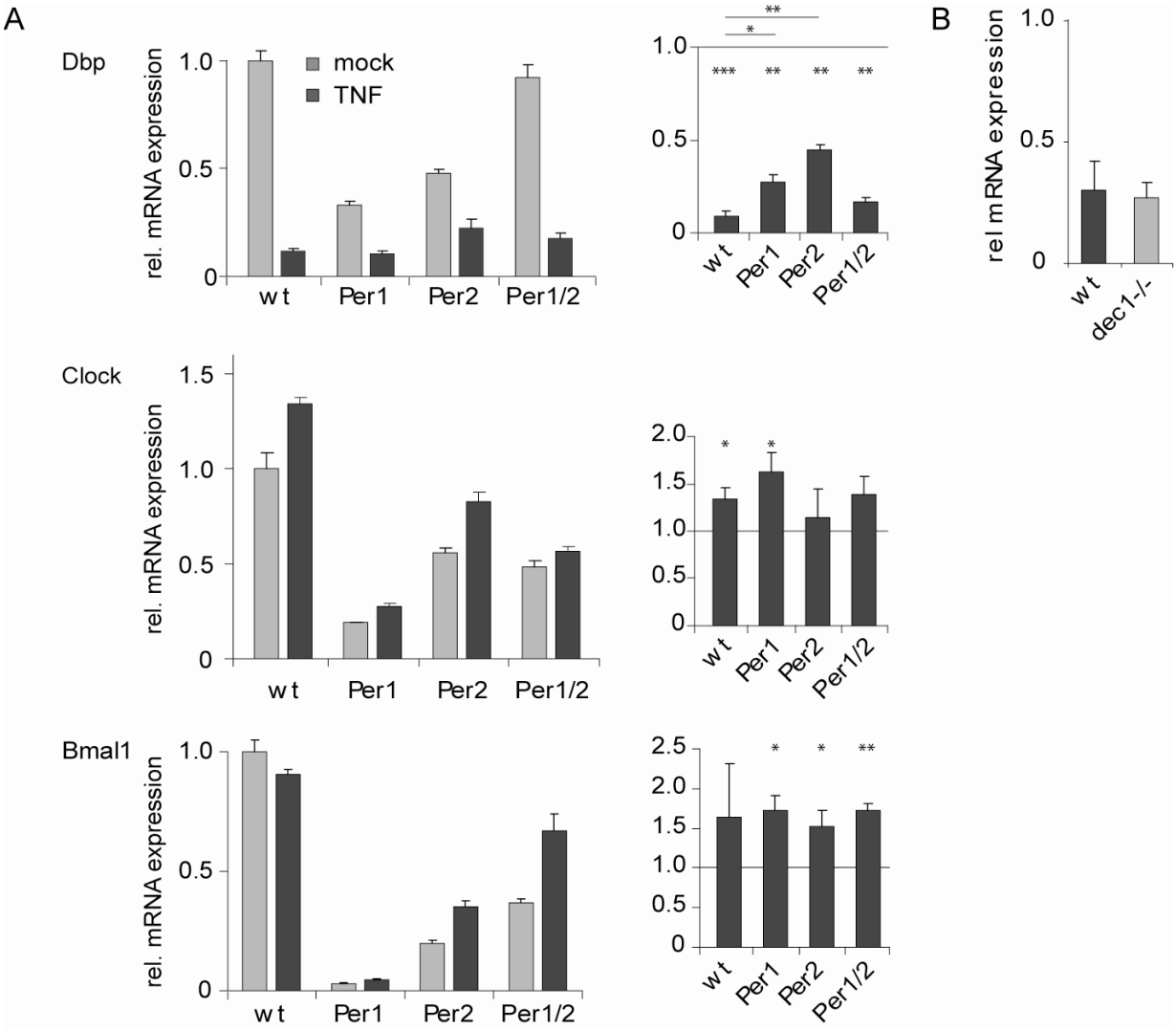


Fig. 3: The transient induction of negative clock regulators by TNF-α is not responsible for the repression of *Dbp*. (A) *Dbp* expression is repressed in wt, *Per1*, *Per2* or *Per1/2* double mutant MEFs, whereas the expression of the central clock genes *Clock* and *Bmal1* remains unchanged. Cells were stimulated with 10 ng/ml TNF-α for 6 h. Shown is the mean + SD; left panel shows 1 representative experiment done in triplicates where the gene expression in wt untreated cells is set to 1; right panel is the mean of 3 independent experiments done in triplicates, where 1 represents the relative expression in the corresponding not TNF-α treated MEFs. (B) *Dbp* inhibition is independent of the presence of DEC1. Human keratinocytes, HaCaT cells, lacking the *Dec1* gene were treated with 10 ng/ml TNF-α for 4 h. Shown is the relative expression compared to untreated cells. Bars represent mean + SD; n=3 in triplicates. For A and B we used paired samples t-test; *, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.0005$.

Signaling via TNFR1 leads to the *Per1* peak and *Dbp* downregulation independent of NF-κB, AP-1 or caspase activation

To analyze the dependency of TNF receptors on the clock gene responses - i.e. *Dbp* downregulation and *Per1* peak - we generated cells from TNFR1 -/-, TNFR2 -/-, and wt mice. Using RT-PCR, TNFR1 mRNA expression was found to be abolished in TNFR1 -/- but still present in TNFR2 -/- MEFs. TNFR2 mRNA expression is detected in both knockout genotypes, because the *tnfr2* deletion is situated outside of the amplified sequence (Fig. 4A). However, cell surface receptor expression is completely abolished in the respective knockouts (Fig. 4B). After 1 h TNF-α stimulation, the *Per1* peak could be detected in wt MEFs similar, although to a lower extent than in NIH 3T3 cells. A *Per1* peak was also seen in TNFR2 -/-, but not anymore in the TNFR1 -/- MEFs (Fig. 4C, left panel). To assay the downregulation of clock-controlled genes we looked at a later time point (6h), where inhibition is complete for all genes usually affected (6). *Dbp* and *Per3* were downregulated in wt and TNFR2 -/-, but not in the TNFR1 -/- MEFs, whereas *Clock* and *Per1* were unaffected in all genotypes (Fig. 4C, right panel). However, it is to note that, for unknown reasons, *Per1* was not downregulated in wt MEFs which is in contrast to the strong inhibition seen in NIH 3T3 cells. Our data show that both the TNF-α induced *Per1* peak at early time points and the inhibition of *Dbp* is mediated by TNFR1.

The major signaling pathway induced by TNFR1 leads to the activation of NF-κB transcription factor by its deliberation from the complex with the inhibitor of κB (IκB) proteins and subsequent translocation to the nucleus. The NF-κB transcription factor is composed mainly of the dimerized partners p65 and p50, transcriptional activity being strictly dependent on p65. Targeted inactivation of the RelA (p65) subunit of NF-κB in mice showed embryonic lethality due to massive liver apoptosis (16). The lethal phenotype could be reversed by breeding the mice in TNF-α deficient animals (17). Here, we analyzed p65 -/- MEFs compared to wt control MEFs of the same genotype. As expected in western blot of p65, p65-/- clearly distinguish from wt MEFs (Fig. 5A, left panel). Further, we analyzed the capability of these cells to activate a 4x-κB site element luciferase reporter gene and IL-6 induction. The p65 -/- MEFs had lower luciferase baseline levels and were not able to induce NF-κB after short TNF-α treatment (data not shown). In addition, $p65^{-/-}$ MEFs lacked the ability

to respond to TNF-α by inducing IL-6 mRNA (Fig. 5A, right panel). Since TNF-α treatment in p65 -/- MEFs leads to apoptosis starting around 4 h we analyzed the effects at time points 1 h (*Per1* peak) and 4 h (*Dbp* repression). In p65 -/- MEFs TNF-α still induced the *Per1* peak and repressed *Dbp* expression (Fig. 5B). This observation is supported by lack of effect on *Dbp* inhibition when adding wedelolactone (data not shown). This compound inhibits NF-κB-mediated gene transcription by blocking the phosphorylation and degradation of IκB.

In addition to NF-κB activation, we also used an inhibitor to the c-jun N-terminal kinase (JNK), which phosphorylates c-Jun. Together with c-Fos, c-Jun forms the transcription factor AP-1. Again, early effects on *Per1* peak and *Dbp* repression at 1 h were unaffected by the inhibition (Fig. 5C). The same holds true for inhibition of caspases with a pan-caspase inhibitor (Fig. S2).

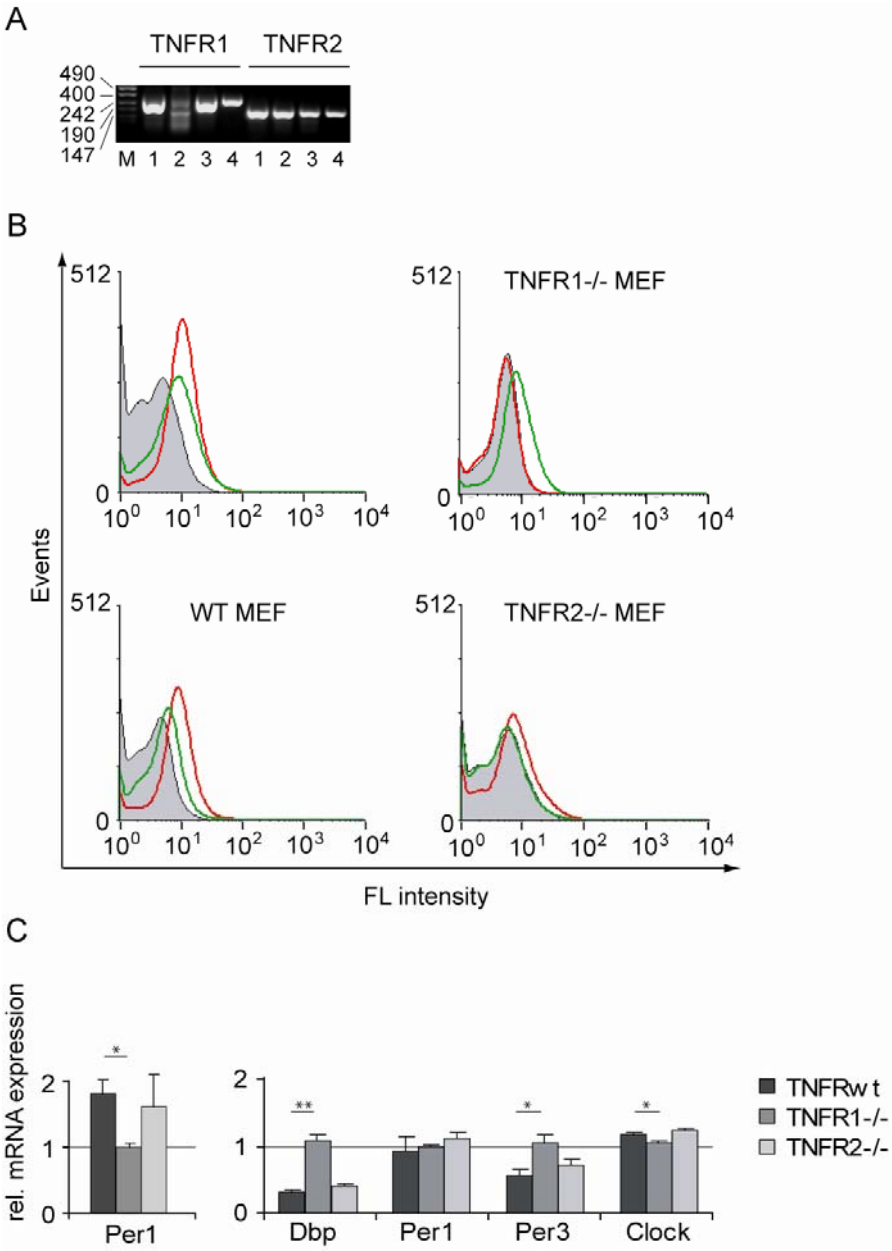


Fig. 4: TNFR1-dependent *Per1* induction and *Dbp* repression. (A) RT-PCR of TNFR1 and TNFR2 in MEFs from wt (1), TNFR1 -/- (2), TNFR2 -/- (3), or peritoneal macrophages (4). (B) Confirmation of cell surface expression of TNF receptors by FACS. B16 murine melanoma cells, wt, TNFR1 -/-, TNFR2 -/- MEFs were used for FACS staining with isotype (grey), TNFR1 (red line), and TNFR2 (green line) antibodies. (C) Different genotype MEFs were treated with 10 ng/ml TNF-α in serum-free medium for 6 h (left panel) or 1 h (right panel). Shown is the mean + SD; n=3 (left panel) or n=4 (right panel) both in triplicates. Paired samples t-test; *, $p \leq 0.05$; **, $p \leq 0.005$.

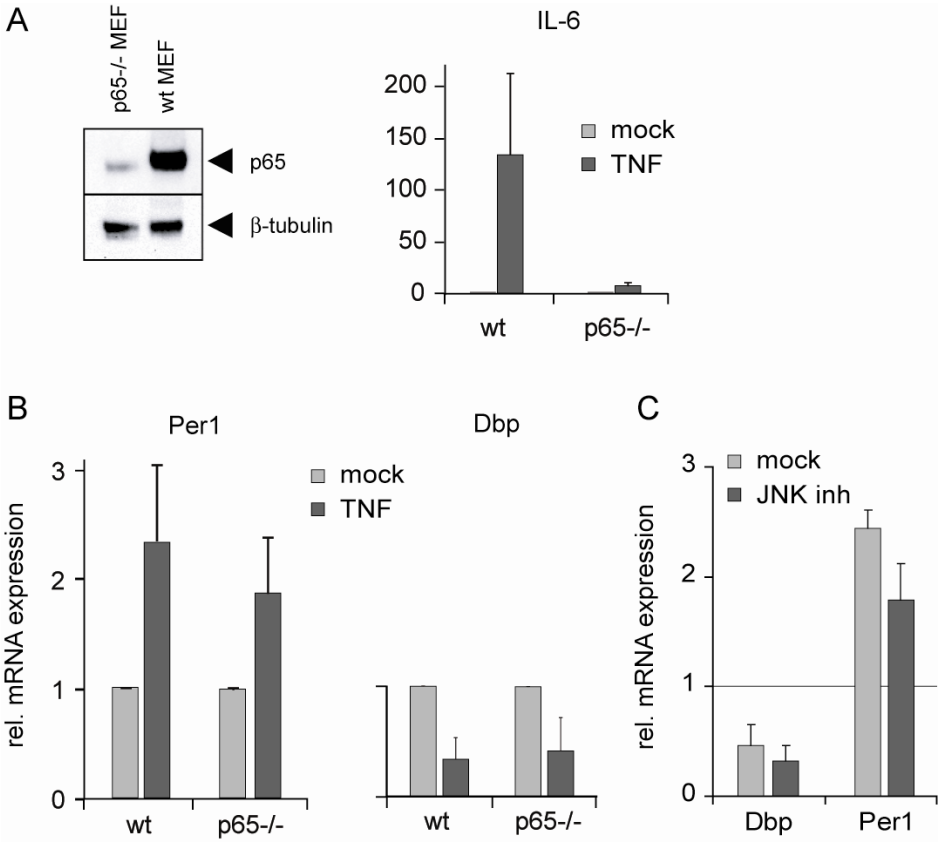


Fig. 5: The c-jun N-terminal kinase and p65 are not involved in the early effects of TNF-α on clock genes. (A) p65 -/- MEFs do not express p65 and fail to induce IL-6 upon TNF-α stimulation. Left panel: wt or p65 -/- MEFs were lysed and subjected to Western blot analysis. b-tubulin was used as a loading control. Right panel: wt or p65 -/- MEFs were treated with 10 ng/ml TNF-α in serum-free medium for 4 h (Dbp panel) or 1 h (Per1 panel). Shown is the mean + SD; n=4 in triplicates. There is no significant differences between wt and p65 -/- MEFs. (C) A JNK inhibitor does not prevent the *Per1* peak nor the *Dbp* repression. NIH 3T3 fibroblasts were pretreated with 1μM inhibitor for 1 h and then stimulated with 10 ng/ml TNF-α for another hour. 1 represents the expression level in untreated cells. n=3 in triplicates.

Per1 peak and *Dbp* inhibition induced by TNF-α are dependent on calcium ion signaling and activation of p38 MAPK

Next we assessed the role of intracellular Ca^{2+} in the TNF-α induced clock gene response. As previously reported, we confirmed that calcium ionophores (ionomycin and PMA) induce *Per1,* but in addition we could also observe a downregulation of *Dbp* at the early phase (data not shown). To further confirm an involvement of intracellular Ca^{2+} signaling in the regulation mechanism, we used the intracellular calcium chelator BAPTA-AM. The addition of BAPTA-AM effectively reduced the *Per1* peak after 1 h treatment of NIH 3T3 fibroblasts with TNF-α. Moreover, BAPTA-AM also effectively blocked the repression of *Dbp.* On the other hand ethylene glycol

tetraacetic acid (EGTA) which chelates extracellular calcium and thereby inhibits influx of extracellular calcium, had only minor effects (Fig. 6A). Given these results, we find TNF-α acting on clock gene expression by modulation of intracellular calcium. In the attempt to elucidate the implication of further TNF-α signaling enzymes, we tested several kinase and phoshatase inhibitors. However, blocking the protein phosphatases (PP) 1 and PP2a or the kinases phosphoinositide-3-kinase, extracellular signal regulated kinase I and II, or calcium/calmodulin dependent kinase II had no influence on clock gene regulation by TNF-α. Solely, the inhibition of the MAPK p38 was able to prevent the *Per1* peak (Fig. 6B). However *Dbp* expression inhibition was not affected.

Taken together, the data suggests that *Per1* induction by TNF-α is dependent on inatracellular calcium and p38 signaling

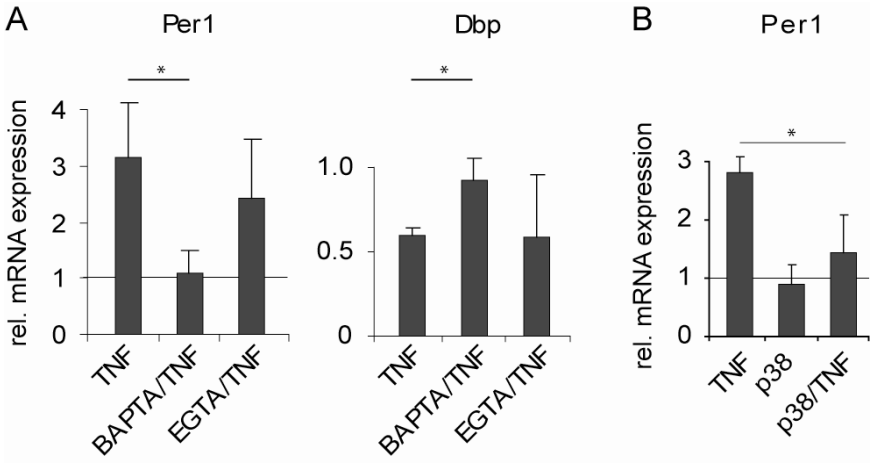


Fig. 6: The inhibition of *Dbp* is mediated by intracellular Ca^{2+} and the induction of *Per1* is mediated by intracellular Ca^{2+} and p38 MAPK. (A) Effect of the intracellular calcium chelator BAPTA-AM on *Per1* induction and *Dbp* inhibition by TNF-α. NIH 3T3 were treated with EGTA (1 µM) or BAPTA-AM (30 µM) 30 min before a 1 h stimulation with 10 ng/ml TNF-α. Expression of untreated samples corresponds to 1. Shown is the mean + SD; n=3-5 in triplicates; (B) Effect of p38 MAPK inhibition on the *Per1* peak induced by TNF-α. NIH 3T3 were treated with SB203580 (10 µM) 30 min before a 1 h stimulation with 10 ng/ml TNF-α. Expression of untreated samples corresponds to 1. Shown is the mean + SD; n=4 in triplicates. For A and B we used paired samples t-test. *, $p \leq 0.05$.

Chromatin remodeling defines clock gene regulations

To analyze the chromatin remodeling on the *Dbp* gene after TNF-α stimulation, we performed chromatin immunoprecipitation (ChIP) assays against acetylated histone

H3. There is clearly less acetylated histone H3 at the *Dbp* E-box 857 corresponding to the less activity in gene transcription. Therefore, we aimed to block deacetylation by using Trichostatin A (TSA) (HDAC 2 und 3) or splitomicin (HDAC 1). We did not find any significant effects on the inhibition of *Dbp* by TNF-α using TSA or splitomicin. These results suggest that active deacetylation is not important for blocking gene transcription of clock genes.

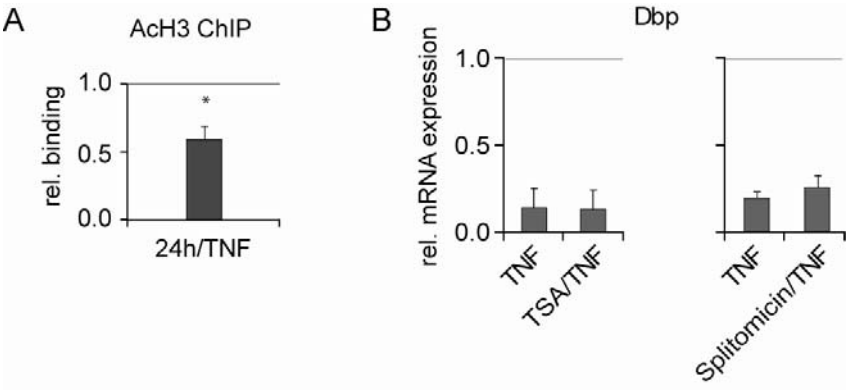


Fig. 7: Less acetylated histone H3 at the *Dbp* E-box 857 corresponds to reduced expression, but active deacetylation is not involved in the inhibition of *Dbp* by TNF-α. (A) Less acetylated histones H3 at the *Dbp* E-box 857 in cells treated with TNF-α assayed by ChIP. NIH 3T3 fibroblasts were synchronized by a serum shock and stimulated with 10 ng/ml TNF-α in serum-free medium. After 24 h, at the peak of *Dbp* gene expression, chromatin was isolated and subjected to ChIP assay. Shown is the relative binding of acetylated histones compared to unstimulated cells. n=3 in duplicates or triplicates. (B) Trichostatin A and splitomicin have no effect on the inhibition of *Dbp* by TNF-α. After a 6 h or overnight pretreatment with TSA (10 ng/ml) or splitomicin (120 µM) NIH 3T3 fibroblasts were stimulated with 10 ng/ml TNF-α for 3 h. 1 is the expression level in mock treated cells. Shown is the mean + SD; n=3 for TSA, n=2 for splitomicin, both in triplicates; For A and B we used paired samples t-test. *, $p \leq 0.05$.

Discussion

The biology of circadian rhythms in mammals is dependent on the appropriate function of the molecular clock within all cells of the body. The activation of the immune system parallels the deregulation of circadian functions during the acute phase of infectious and autoimmune diseases; it is therefore intriguing to speculate that this may be linked to the sickness behavior syndrome. Recently, TNF-α was found to impair locomotor activity in mice and to impair E-box driven clock gene expression (6). Fibroblasts treated for 24 h with TNF-α respond with a decrease of E-

box mediated transcription of *Dbp*, *Tef* and *Hlf* and of *Period 1-3*. The data on the effect of TNF-α on fibroblasts presented here, show that TNF-α treatment for only 1 h induces *Per1*. This effect was not associated with synchronization of the fibroblasts (data not shown), although a striking characteristic of the synchronization of cells is the induction of the clock gene *Per1* mRNA and protein. This may be a necessary event for the induction of circadian gene expression, but it is probably not sufficient as shown for epidermal growth factor which strongly induces *Per1* but still does not induce circadian gene expression (18).

Surprisingly, we found that besides *Per1,* TNF-α can also transiently induce other negative elements of the clock such as *Per2*, *Cry1* and *Dec1*. Because blockade of *de-novo* protein synthesis partially reverses *Dbp* inhibition, the increase in negative regulators could be partially responsible for TNF-α mediated impairment of *Dbp* expression. However, when assaying *Per1*, *Per2*, and *Dec1* deficient cells, we have not found direct evidence for this hypothesis. *Dec1* and *Dec2* have been reported to be regulated by several cytokines and *Dec1* is also induced by TNF-α in our *in-vitro* setting (19). Even though we did not analyze the effect of *Cry*, there is indirect evidence that also *Cry* is not responsible for TNF-α induced downregulation of *Dbp* expression, because the heterodimerization of CRY:PER proteins should also be impaired in our *Per* deficient fibroblasts. Furthermore the induction of *Cry1* expression appears fairly late when *Dbp* is already strongly inhibited. These data strongly suggest that neither *Per1* and *Per2*, nor *Dec1* and *Cry1* are involved in *Dbp* repression. However, it still cannot be excluded that in *Per1/Per2* double knockout cells *Dec1* may lead to suppression of *Dbp* expression. Moreover, *Per1* and *Per2* may compensate for *Dec1* deletion.

TNF-α mediates its function by TNF receptors, TNFR1 and TNFR2. By using knockout fibroblasts, we observed that signaling via TNFR1 is sufficient to detect the early *Per1* upregulation and early as well as late *Dbp* downregulation. There are evidences that soluble murine TNF-α has a higher affinity to TNFR1, and that ligand binding to TNFR2 is short and unstable (20). We detected no cell activation by measuring IL-6 expression in TNFR1 deficient cells, still expressing TNFR2 on the surface, indeed emphasizing the higher relation of soluble TNF-α to TNFR1. We were surprised to find that the main TNF-α signaling pathways, comprising the NF-κB, JNK and caspase pathways are not responsible for clock gene regulation and that rather a

"side" pathway, the Ca^{2+} signaling, is mediating the E-box dependent effect of TNF-α. A role for calcium signaling in circadian clocks has been extensively studied in the SCN. There it has been shown that not only Ca^{2+} can regulate clock gene expression but also that the circadian clock controls intracellular Ca^{2+} rhythms (21-23). Ca^{2+} influx is an initial cellular event in response to glutamate stimulation by the retinohypothalamic tract in the SCN, thereby disturbing the intracellular Ca^{2+} homeostasis. It has therefore been proposed to be one of the messengers conveying environmental time signals to the endogenous clock. The *Per1* gene is first induced before it is inhibited at later time points. This induction is dependent on the activation of p38 and intracellular Ca^{2+}. These to messengers are well described to lead to a phosphorylation of the cAMP responsive element (CRE) binding protein (CREB). In the SCN, the binding of CREB to CRE sites in the *Per1* promoter in turn induces gene expression (24, 25). Although it remains to be further investigated, we think that *Per1* expression is first induced through its CRE sites and thereafter inhibited through its E-box sequences. Here, we report the novel finding of clock gene expression regulation by Ca^{2+} signaling outside of the SCN. It therefore seems that Ca^{2+} plays a central role in the periphery as well. We think that analogue to the SCN, Ca^{2+} could also be considered as a physiological sensor in the periphery, transmitting immunological changes to the molecular clock and thereby coordinating the special needs to specific time windows.

Materials and Methods

Cell lines, cytokines and chemicals

Murine fibroblast cells, NIH 3T3 (CRL-1658) were obtained by the American Type Culture Collection; human keratinocyte cells, HaCaT, knockout for *Dec1*, were generously provided by B. Vogelstein (9). Embryos of wt (C57Bl/6), TNFR1-/-, TNFR2-/- mice were used at day 12 of gestation (E12). The liver and heads were removed, the remaining tissue was digested with 0.25% trypsin-EDTA in DMEM for 30 min at room temperature. The dissociated cells were plated in DMEM containing

20% FCS. They were split 1:4 until they reached passage 10. Cells were used within passage 10 - 30. *p65-/-* and control MEFs were generously provided by M. Hottiger (University of Zürich) (10). *mPer1-/-* and *mPer2-/-* single knockout and *mPer1-/-;mPer2-/-* double knockout MEFs were generously provided by U. Albrecht (University of Fribourg) (11).
Recombinant murine (rm) TNF-α, was purchased from Peprotech (London, U.K.); BAPTA-AM, Cycloheximide, EGTA, splitomicin, actinomycin D from Sigma (ST.Louis, MO, USA); SB203580 from EMD chemicals (Darmstadt, Germany) and Trichostatin A from InvivoGen (St. Diego, USA).

Cell culture and stimulation

NIH 3T3 fibroblasts were grown in DMEM (Gibco, Basel, Switzerland) supplemented with 10% FBS (PAA Laboratories, Pasching, Austria) and Glutamax (Gibco). For stimulation cells were grown to confluency and then medium was replaced with serum-free DMEM/Glutamax with or without TNF-α. After stimulation, tissue culture plates were washed once with ice-cold PBS solution and kept at –70 °C until the extraction of whole-cell RNA.

FACS staining

Cells were trypsinized and resuspended in FACS buffer (PBS, 0.5% BSA, 0.02% Sodium azide) at 4°C. Antibody staining was performed with 1:100 isotype-ab (553951, BD Pharmingen), TNFR1-ab (559915, BD), TNFR2-ab (559916, BD) followed by 1:200 secondary biotinylated Goat anti-Hamster antibody (127-065-160, jackson immunoresearch), then with 1:200 SAV-APC (554067, BD). Staining was analyzed with FACS analyzer (Partec, Germany). Curves were smoothed for better visualization of the results.

Western blot

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA 1 mM PMSF, 1% NP40, 10% glycerol, protease inhibitors) and protein concentration

was measured using BCA (Pierce). 20 µg of total proteins were run with Tris-Tricine SDS-PAGE (12). Western blotting was performed at 4°C and blot was blocked with 5% milk powder (Biorad) in TBS-T overnight. Staining was performed with 1:5000 p65 antibody (sc-372 X, santacruz), and 1:100'000 goat anti-mouse-HRP ab (jackson immunoresearch), and ECLplus (Amersham) reaction. Same blots were re-stained with 1:2000 β-tubulin ab (Abcam).

RNA isolation and gene expression analysis

Whole-cell RNA from cultured cells was extracted using TRIzol (Invitrogen) or peqGOLD RNAPure (peqLab) according to the manufacturer's instructions. Subsequently, RNA was reverse-transcribed using random hexamers (Roche) and M-MuLV reverse transcriptase (Applied biosystems). The cDNA equivalent to 20 ng of total RNA was PCR-amplified in an ABI PRISM HT7900 detection system (PE-Applied Biosystems) using the TaqMan Universal PCR Master Mix (Applied Biosystems) and quantified as follows. Primers and probes for Taqman analysis were either purchased from Applied Biosystems or purchased from Microsynth, Balgach Switzerland as described in detail in SI table 1. The relative levels of each RNA were calculated by the $2^{-\Delta\Delta Ct}$ method (Ct standing for the cycle number at which the signal reaches the threshold of detection); *Gapdh* mRNA was used as a housekeeping gene. Each Ct value used for these calculations is the mean of two duplicates of the same reaction. Relative RNA levels are expressed as x-fold variations compared to untreated.

Chromatin immunoprecipitations

All steps were performed at 4 °C or on ice. About 1 $x10^7$ cells per condition were used as starting material. Cells were fixed for 5 min at 37 °C with 1x PBS containing 1% formaldehyde. Subsequently, cells were washed twice with ice-cold 1x PBS and scraped in Glycin buffer (125 mM Glycine; 100 mM Tris, pH 9.4; 10 mM DTT). To stop fixation, cells were kept on ice for 15 min. After a short spin of 1,5 min, 2000 g, pelleted cells were resuspended in buffer I (0.25% Triton x-100; 10 mM EDTA; 1 mM EGTA; 10 mM HEPES, pH 6.5) by vortexing. Cells were pelleted at 2000 g, 5 min and carefully resuspended in buffer II (200 mM NaCl; 1 mM EDTA; 1 mM EGTA; 10 mM HEPES, pH 6.5). After another centrifugation for 5 min, 2000 g, nuclei were carefully

resuspended in 190 µl nuclei resuspension buffer at RT (165 mM NaCl; 2.2 mM EDTA; 22 mM Tris-HCl, pH 8). After addition of 10 µl 20% SDS, nuclei were submitted to 5 x 1 min sonication steps with 30 sec pauses in between using the Bioruptor™ sonication machine (Diagenode, Liège, Belgium). The fragmented cross-linked chromatin was then diluted tenfold in dilution buffer (1.1% Triton X-100; 2 mM EDTA; 150 mM NaCl; 20 mM Tris-HCl, pH 8). 100 µl chromatin was used as starting material for DNA quantification experiments and 1 µl was used as input measurement. Immunoprecipitated DNA fragments captured by protein A–agarose/salmon sperm DNA (Millipore, Billerica, MA, USA) were washed sequentially with 500ml TSE I (20 mM Tris-HCl, pH 8; 150 mM NaCl; 2 mM EDTA; 0.1% SDS; 1% TX-100), TSE II (20 mM Tris-HCl, pH 8; 500 mM NaCl; 2 mM EDTA; 0.1% SDS; 1% TX-100), buffer III (0.25 M LiCl; 1% NP-40; 1% DOC; 1 mM EDTA; 10 mM Tris-HCl, pH 8) and TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA) and the cross-links, including inputs, were reversed overnight at 65 °C in 20 mM Tris-HCl, pH 7.5; 150 mM NaCl; 2 mM EDTA; 1% SDS. DNA fragments were recovered using the DNA columns from the Qiagen MiniPrep kit according to the manufacturers protocol. Two microliters of each reaction were directly used in 10 µl real-time PCR reactions using a 7900 HT machine (Applied Biosystems). The amount of DNA was quantified as described above. Each value was normalized to the corresponding input. Three independent experiments were done in duplicate or triplicate. The primers and Taqman probes used are listed in table 1. The antibody against acetylated histone H3 was purchased from Millipore.

Nascent transcript assay

All steps were performed on ice or at 4 °C. 2 x10^7 cells were scraped and washed twice with PBS, resuspended in 400 µl of HB 0.3 M sucrose (10% glycerol, 0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM HEPES, pH 7.9, 0.5 mM EDTA, 0.15 mM spermine, 0.5 mM spermine, 0.5 mM PMSF, 1 mM DTT) and lysed by the addition of 400 µl HB 0.3 M sucrose containing 0.8% NP-40. After a 10 min incubation, nuclei were pelleted through a 1.2 ml cushion of HB 0.9 M sucrose by a 10 min centrifugation at 1500 g and resupended in 500 µl of nuclear suspension buffer (75 mM NaCl, 20 mM Tris-HCl, pH 7.9, 0.5 mM EDTA, 0.125 mM PMSF, 0.85 mM DTT,

50% glycerol, 100 mg/ml tRNA). Nuclei were lysed on ice for 10 min in 2400ml nuclear lysis buffer (0.3 M NaCl, 20 mM HEPES, pH 7.6, 0.2 mM EDTA, 7.5 mM MgCl2, 1 M urea, 1 mM DTT, 1 % NP-40 and 100 mg/ml tRNA). Chromatin was pelleted by 10 min at 15,000 g in a microfuge, resuspended in 500 ml of chromatin suspension buffer (50 mM Na acetate, pH 5, 50 mM NaCl, 0.5% SDS, 100 mg/ml tRNA), extracted three times with hot phenol mix (acid phenol saturated with water; 5 mM Na acetate, pH 5; 5 mM NaCl) at 65 °C. After addition of NaCl to a final concentration of 150 mM, nucleic acids were precipitated with ethanol, resuspended in 50 ml 10 mM Tris-HCl, pH 7.5, and treated with RNase-free DNase to eliminate genomic DNA. Nascent transcripts were then extracted with phenol-chloroform, precipitated and resuspended in 30 µl of 10 mM Tris-HCl, pH 7.5. 10 µg were used for reverse transcription and real-time PCR, which were performed as described above. Results were corrected for cDNA input by amplifying nascent gapdh transcripts. The primers and Taqman probes used are listed in table 1.

Footnotes

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The abbreviations used are: tumor necrosis factor, TNF; D site albumin promotor binding protein, Dbp; nuclear factor-kappaB, NF-κB; activator protein-1, AP-1; mouse embryonic fibroblasts, MEF;

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Supporting information

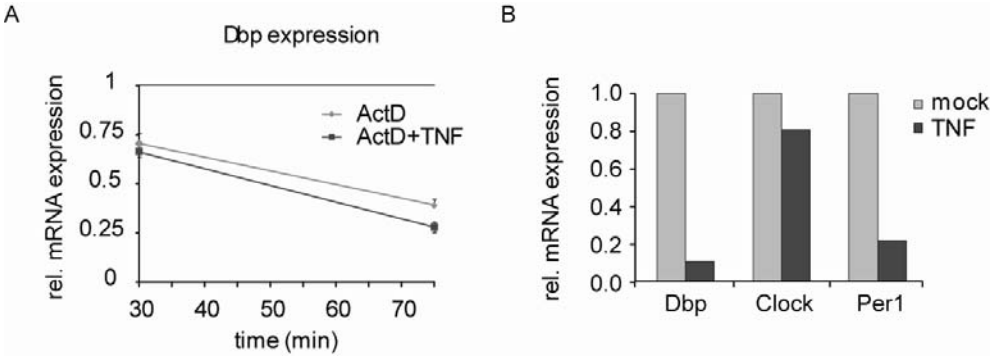

Fig. S1: The reduced levels of *Dbp* mRNA in NIH 3T3 fibroblasts is not due to an accelerated mRNA degradation. (A) *Dbp* mRNA degradation is very rapid. Cells were treated with 1 µg/ml Act D with or without 10 ng/ml TNF-α and collected after 30 or 75 min for mRNA quantification. Values are relative to untreated samples collected at time point 60 min. n=1 in triplicates. (B) Cells were stimulated with 10 ng/ml TNF-α overnight or left untreated. RNA was prepared for nascent transcripts analysis. n=1 with one sample per condition.

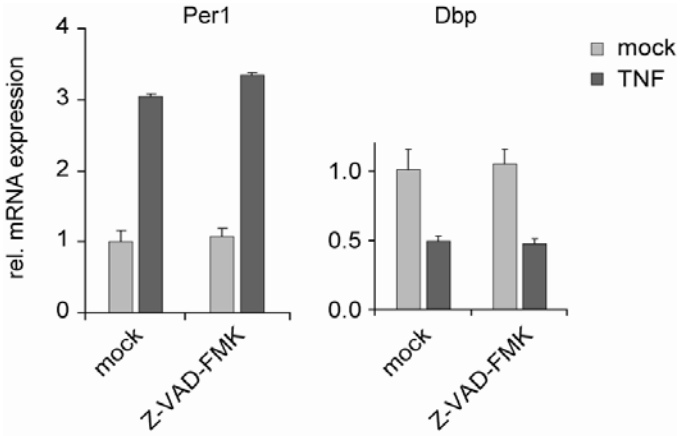

Fig. S2: Caspases are not involved in *Per1* and *Dbp* regulations. NIH 3T3 cells were treated with 10 ng/ml TNF-α with or without 50μM Z-VAD-FMK simultaneously for 1 h. Values are relative to samples without TNF-α stimulation. Shown is one experiment done in triplicates.

Assay	Gene	FP	Probe	RP
nascent transcripts	*clock*	GGGATGGGTGGGTAATGTCA	CAGAGAGTATTGGTACACAGGACAGCGTAGCA	ACTACAGTTCTTAGGCGCACAGATG
	dbp	CTACGTTCCCGGATCCTACAC	CCCATTCCCCAAGTGGATGGTGTT	GATCCCGATATTCTCTTCTGCAA
	per1	ACCAGCCATTCCGCCTAAC	CTGCCTACTCATTGCCGAGCG	CCTAGCCTATCCACCTTCATAACC
ChIP	*Dbp 857*	ATGCTCACACGGTGCAGACA	CCTAGTTTCCATGTGACCCTGCGAGG	CTGCTCAGGCACATTCCTCAT
PCR	*tnfr1*	ACCAAGTGCCACAAAGGAAC		CACGCACTGGAAGTGTGTCT
	tnfr2	TACCAAGGGTGGCATCTCTC		TCCTGGGATTTCTCATCAGG
real time PCR	*dbp*	GCGAGAAGTGCAAAATTGGC	CGCGCGCCTGTGTCCCTTG	CGGGAGGCTCCTATAGTCTGG
	clock	TTGCTCCACGGGAATCCTT	ACACAGCTCATCCTCTCTGCTGCCTTTC	GGAGGGAAAGTGCTCTGTTGTAG
	cry1	CTGGCGTGGAAGTCATCGT	CGCATTTCACATACACTGTATGACCTGGACA	CTGTCCGCCATTGAGTTCTATG
	cry2	TGTCCCTTCCTGTGTGGAAGA	CAGTCACCCTGTGGCAGAGCCTGG	GCTCCCAGCTTGGCTTGA
SYBR green	*per1 1A*	TTACTCTGGAGCCGTCGAACTTGT		AGGACGAAACAGGGAAGGTGAAGA
	per1 1B	GAACGGCCAGGTGTCGTGATTAAA		AGGACGAAACAGGGAAGGTGAAGA
		Pre-developed taqman assays (Applied biosystems)		
real time PCR	*gapdh*	4352339E		
	per1	Mm00501813_m1		
	per2	Mm00478113_m1		
	per3	Mm00478120_m1		
	dec1	Mm00478593_m1		
	dec2	Mm00470512_m1		
	IL-6	Mm00446190_m1		
	human gapdh	0-0702011		
	human dbp	Hs00609747_m1		

Table S1. Sequences for primers and probes.

5. GENERAL DISCUSSION AND OUTLOOK

We found that the pro-inflammatory cytokine TNF-α impairs the molecular circadian clock *in vivo* and *in vitro*. By applying the cytokine constantly to mice with the use of subcutaneously implanted osmotic minipumps, we could observe a decrease in the mRNA levels of several central clock genes, such as *Per1*, *Per2*, *Per3*, and clock output genes, such as *Dbp*, *Tef* and *Hlf* in the liver. The expression of the core clock transcription factors *Clock* and *Bmal1* remained unchanged. In addition, these mice showed less locomotor activity and an increase in rest periods indicating that these mice may suffer from fatigue. In NIH 3T3 fibroblasts, which are used as a model to study circadian rhythms, we assessed clock gene expression in the presence of recombinant TNF-α. In line with the results from the *in vivo* experiments, the three *Period* clock genes and the three *PAR-bZIP* output genes were also affected *in vitro*. We therefore decided to continue using this *in vitro* model for further analysis of the underlying mechanisms. We noticed that genes bearing an E-box in their promoter seem to be the ones targeted by TNF-α, whereas *Clock* and *Bmal1* that are devoid of E-boxes are unaffected. To confirm the hypothesis that TNF-α acts indeed via these E-boxes we used luciferase reporter constructs, thereby showing that luciferase controlled by E-box elements is inhibited in the presence of the cytokine. When these E-boxes were mutated *Dbp* was de-repressed. Complementary to that, when luciferase is driven by the *Bmal1* promoter no change could be observed after addition of TNF-α. Interestingly, the total inhibition is less dramatic in the luciferase assays compared to RNA expression analysis. This could be an indication for a supplementary inhibition mechanism independent of E-boxes.

Several scenarios can be envisaged that could lead to an E-box dependent down-regulation.

1) Induction of an inhibitor that would either bind directly to the E-box thereby preventing CLOCK/BMAL1 binding or bind to the CLOCK/BMAL1 complex and disable transactivation. This inhibitor may be synthesized upon TNF-α stimulation or could also already be present in the cytoplasm and would be shuttled to the nucleus after stimulation with TNF-α. Experiments in which we used the translation inhibitor cycloheximid would support both possibilities. After one hour stimulation, *Dbp*

expression was partly de-repressed in the presence of the inhibitor. We think that the rapid inhibition can take place without the need of a newly synthesized protein and that at later time points an inhibitory protein needs to be produced to emphasize the dampening. The early and late effect may be mediated by the same promoter elements but could also act on two different regions again supporting the notion that two different inhibition mechanisms could be implicated.

The most obvious possible inhibitors would be members of the negative limb of the molecular clock. Although MEFs with single *Period* gene knock-out had little but significant effect on *Dbp* expression, MEFs lacking both genes still showed a comparable downregulation to wild-type MEFs.

Since *Dec1* and *Dec2* have been reported to be regulated by several cytokines and because *Dec1* is also induced by TNF-α in our *in vitro* setting, it was an additional strong candidate inhibitor (138). However, we could not find an implication for this gene.

The induction of *Cry1* expression appears fairly late when *Dbp* is already strongly inhibited, nonetheless, it would still be worth investigating for a role of this gene in the inhibitory mechanism. Since the degradation of CRY1 could be inhibited, the protein would accumulate and inhibit CLOCK/BMAL1 complexes. The same scenario could apply to CRY2.

As mentioned above, only the localization of the inhibitor could be altered by TNF-α. Therefore, it would be a possibility to analyze the localization of the negative regulators by Western blot or Immunfluorescence imaging. In addition, to define whether the import of one of these negative regulators is an important event in TNF-α mediated clock gene expression dampening, it would be elegant to prevent the translocation into the nucleus. Overexpression of negative regulator proteins that are mutated in their nuclear import signal sequences would prevent nuclear import in a dominant negative way.

2) Posttranslational modifications of CLOCK and/or BMAL1 would lead to differential stability, localization or DNA affinity of the proteins (139). It will be important to analyze the CLOCK and BMAL1 protein levels as well as their modifications and localization. After separating the nuclear from cytoplasmic protein fraction the amount of CLOCK and BMAL1 could be quantified by Western blot. An accumulation of BMAL1 for instance would lead to BMAL1 homodimer formation which are not

transcriptionally active. On the other hand, a decrease of CLOCK protein could approach the phenotype of *Clock* knock-out mice. These animals show a decreased expression of *Clock* and clock controlled genes, such as *Per1*, *Rev-erbα* and *Dbp* in the liver, hence, a similar picture to TNF-α treated mice (140).

Western blot analysis could additionally reveal possible modifications evoked by the cytokine. Phosphorylation residues make the protein run slower on a gel thereby the native and modified proteins are uncovered. Immunoprecipitation of the proteins and subsequent analysis by mass spectrometry could deliver further details like the localization of the phosphorylated residues. This is important as the outcome in activity may be different (58, 67).

Differential DNA binding activities of the two core proteins CLOCK and BMAL1 could be analyzed by chromatin immunoprecipitation. A scenario where CLOCK and BMAL1 show reduced DNA binding activity in the presence of TNF-α would speak for an inhibitor displacing the heterodimeric transcription factor. If there is no change in DNA binding, the transactivation ability may rather be altered by posttranslational modifications.

Although reports about an activation of HATs by TNF-α are missing, it is also conceivable that TNF-α signaling could lead to an enhanced acetylation of BMAL1 and/or PER2. Both modifications would lead to a dampening of E-box dependent transcription.

Finally, due to the impact of BMAL1 sumoylation on its activity, it would be interesting to look for sumo modification after TNF-α treatment. This is of great interest because TNF-α signaling has already been implicated in desumoylation processes. The group of Wan Ming showed that upon TNF-α stimulation of resting endothelial cells, the nuclear protein homeobox-interacting protein kinase 3 (HIPK-3) is desumoylated by the SUMO-1 specific protease SENP1, thus targeting HIPK-3 to the cytoplasm (141, 142). Such a scenario would also be feasible for BMAL1, which has been shown to undergo SUMO modifications.

3) Loss of histone acetylation would lead to a reduction in transcriptional activity. We could not find an implication of HDACs in the inhibitor mechanism but still observed reduced levels of acetylated histones in the presence of TNF-α. This suggests rather a passive deacetylation process. Histones are supposedly no longer acetylated as a consequence of a lack of gene expression activity or a reduced activity of HATs, such

as CLOCK and/or p300. In parallel, histones are regularly exchanged thereby resulting in a lower concentration of acetylated histones over time (143).

We also observed that TNF-α only dampens the peak expression thereby leaving the period length intact. Additionally the effect of the cytokine on *Dbp* expression is very direct, rapid and transient as it can be reversed after removal of the cytokine. In contrast the inhibition of the *Period* genes is delayed. This let us hypothesize that DBP may play a role in *Period* down regulation. Indeed, DBP binding elements have been found in the *Per1* promoter and DBP together with CLOCK and BMAL1 has been described to cooperatively induce *Per1* transcription. A luciferase construct containing 1.3 Kbp of the *Per1* promoter was shown to be induced up to three times more when DBP was co-transfected in addition to CLOCK and BMAL1 (144). Furthermore the phase of *Dbp* expression *in vivo* is slightly earlier to *Per1*. In this context the rapid down regulation of *Dbp* would passively lead to an inhibition of *Per1* through the lack of DBP transcriptional co-activator. Due to their homology to *Per1*, it is thinkable that *Per2* and *Per3* are regulated similarly. It is possible to test this hypothesis by overexpressing DBP. A construct coding for the *Dbp* mRNA under the control of a promoter that is insensitive to TNF-α could be expressed in fibroblasts. DBP quantity would no longer be affected by TNF-α treatment and *Per1* inhibition, if regulated by DBP, would not occur anymore.

An important result was that the effect of TNF-α can be mediated by the TNFR1. This finding may explain the ubiquitous ability of TNF-α to downregulate clock gene expression, as TNFR1 is known to be expressed on almost all cell types (123). Indeed, we analyzed clock gene expression *in vivo* in many different tissues of TNF-α infused mice and always observed an inhibition of most E-box controlled clock genes. *In vitro*, we not only measured a *Dbp* inhibition in NIH 3T3 cells but also in MEFs, human keratinocytes, CAD neurons and the SCN cell line SCN2.2 (data not shown).

Since TNF-α binding to its receptors leads to the activation of a broad range of kinases, we wanted to elucidate which kinase could be involved in the downregulation mechanism. We therefore used inhibitors against kinases either known to be part of the TNF-α signaling cascade or to be involved in clock gene

expression regulation. The only inhibitor interfering with TNF-α mediated clock gene regulation was an inhibitor against the MAPK p38. Inhibiting this kinase in fibroblasts abolishes *Per1* induction by TNF-α. Interestingly inhibitors interfering with the main TNF-α signaling pathways, comprising the NF-κB, JNK and caspase pathways were not able to de-repress *Dbp* expression or abolish the *Per1* peak.

The common pathway leading to *Dbp* repression and early *Per1* induction seems to be the intracellular Ca^{2+} pathway.

Calcium is a widely used messenger that can control several different cellular processes the two main ones being exocytosis and gene transcription. Calcium can enter the cytosol from two different compartments, which are the intracellular organelles where the endoplasmatic reticulum (ER) is the major source, and the extracellular space. Ca^{2+} release from the ER by extracellular stimuli can be provoked by the generation of several different second messengers that target Ca^{2+} channels. The most widely used is inositol 1,4,5-triphosphate (IP3) that activates Ca^{2+} stores by binding to its receptor IP3R. A second Ca^{2+} sensitive channel known to release Ca^{2+} stores from the ER is the ryanodine receptor (RYR). This receptor can be activated by cyclic adenosine diphosphate-ribose (cADPR). The released Ca^{2+} in turn can directly activate both channels and further amplify the signal (145).

Although the contribution of cytosolic Ca^{2+} signaling in the regulation of circadian clock has been extensively studied in a variety of organisms no commonalities in the Ca^{2+}-mediated signaling process have been described among the various circadian clock systems (146). Most studies in mammals were performed in the SCN. There it has been shown that not only Ca^{2+} can regulate clock gene expression but also that the circadian clock controls intracellular Ca^{2+} rhythms (147-149). An exact mechanism is still missing but it is hypothesized that cADPR is an intermediate messenger (146). Furthermore a central role for cAMP has been confirmed recently (149). Ca^{2+} influx is an initial cellular event in response to glutamate stimulation by the RHT in the SCN, thereby disturbing the intracellular Ca^{2+} homeostasis. It has therefore be proposed to be one of the messengers conveying environmental time signals to the endogenous clock, where the RYR dependent Ca^{2+} influx is thought to amplify the signal (150, 151). Ca^{2+}, in turn, phosphorylates cAMP response element-binding protein through activating CaMKII and/or MAPK (152, 153). Subsequently

CREB binds to the CRE elements in the *Per* promoter and induces gene transcription. In our setting it is possible that p38 is activated by Ca^{2+} and then phosphorylates CREB and induces *Per1* via its CRE sites. Besides *Per1*, only CLOCK has been reported to be regulated by Ca^{2+}. Phosphorylation of CLOCK is mediated by the Ca^{2+} dependent PKC pathway leading to its translocation to the nucleus (68). The expression of further clock genes has not been analyzed so far. We are the first to report clock gene expression regulation by Ca^{2+} signaling outside of the SCN. How the repression is achieved is unclear but one possibility could involve cAMP and/or cADPR increase by TNF-α.

TNF-α as well as other pro-inflammatory cytokines have been shown to increase intracellular Ca^{2+} via an increase in cADPR in airway smooth muscle cells (SMC) (154). In these cells sarcoplasmic Ca^{2+} can be released through IP3R channels as well as RYR channels. There is strong evidence that the cell surface protein CD38 is responsible for the TNF-α mediated increase in cADPR. The up-regulation of CD38 mRNA, involving MAPK and transcription factors NF-κB and AP-1, enhances the production of cADPR and thereby Ca^{2+} release. Other cytokines, like IL-13, IL-1β and IFN-γ were also shown to induce CD38 transcription although in a less extensive way. With all these cytokines the intracellular Ca^{2+} release in SMC could be inhibited with the addition of a cADPR agonist, 8-bromo-cADPR (155, 156). It is strongly conceivable that a similar mechanism applies to NIH 3T3 fibroblasts and that the effect of TNF-α on clock genes may partly be mediated by CD38. If indeed TNF-α acts via these Ca^{2+} stores then clock gene expression would be de-repressed in the presence of 8-bromo cADPR.

On the other hand, TNF-α can also directly activate several kinases that have been implicated in IP3R dependent Ca^{2+} release (145, 157). Inhibition of Sphingomyelinase, PKA, PKC or PLC may also show a de-repression in clock gene expression.

A speculative but possible Ca^{2+} dependent inhibition mechanism could be that TNF-α first rapidly induces Ca^{2+} release via IP3R. In parallel, via the NF-κB and AP-1 pathway, CD38 is induced and cADPR synthesized. cADPR then binds to RYR enhancing the Ca^{2+} release from intracellular stores (Figure 2). This pathway is much slower as it comprises the synthesis of a new protein. Our results support such a

model in many aspects. A rapid Ca^{2+} release first induces *Per1* via its CRE sites and inhibits *Dbp*. Additional Ca^{2+} is then also capable of repressing other E-box dependent clock genes such as the *period* genes. This would mean that *Dbp* is more sensitive to calcium changes than *period* genes are. The reason why we were not able to show an implication of NF-κB and/or AP-1 could be because of the short stimulation times we used. According to this model NF-κB and AP-1 would only be involved at later time points. It may also be worth blocking both pathways simultaneously. As mentioned above experiments with cycloheximide, showing a partial de-repression of *Dbp* after 1 hour stimulation, support the idea of a two phase model, where a long term inhibition needs the synthesis of a protein

It will be interesting to monitor the intracellular Ca^{2+}, cADPR and cAMP levels in the NIH 3T3 fibroblasts in the presence or absence of TNF-α.

A further interesting subject of investigation would be the regulation of circadian gene transcription by Ca^{2+}. So far, as mentioned above, only Ca^{2+} dependent *Per1* induction via CREB phosphorylation has been described. We examined the involvement of the calcium/calmodulin kinase II in the inhibitory mechanism but could not find an implication. Similarly a role for the calcium/calcineurin sensitive phosphatase PP1 could be excluded (data not shown). A third well-known calcium dependent enzyme is calcineurin. An implication of this phosphatase remains to be elucidated.

Of particular interest are the calcium sensors S-100 and calmodulin. These two proteins, when loaded with Ca^{2+}, have been reported to bind to bHLH proteins, thereby blocking their DNA binding and/or prevent their post-translational modifications (158-161). Although reports about interaction of these sensors with the bHLH proteins CLOCK and BMAL1 are missing, it is conceivable that the interaction of CLOCK/BMAL1 heterodimers with E-boxes is abolished through the binding of such a calcium sensing protein after TNF-α stimulation. It would therefore be interesting to analyze CLOCK and BMAL1 binding to E-boxes by ChIP assay and an interaction of these two proteins with calmodulin or S-100 in the presence of TNF-α by immunoprecipitation and subsequent Western blot analysis.

Another calcium sensor, downstream responsive element antagonist modulator (DREAM), has been shown to inhibit gene transcription in a Ca^{2+} dependent manner

by binding to DRE sites (162). It is therefore also a candidate that may be involved in clock gene inhibition and needs to be examined.

It is possible that S-100 and calmodulin account for the E-box mediated inhibition, whereas DREAM may provide further repression independent of the E-boxes.

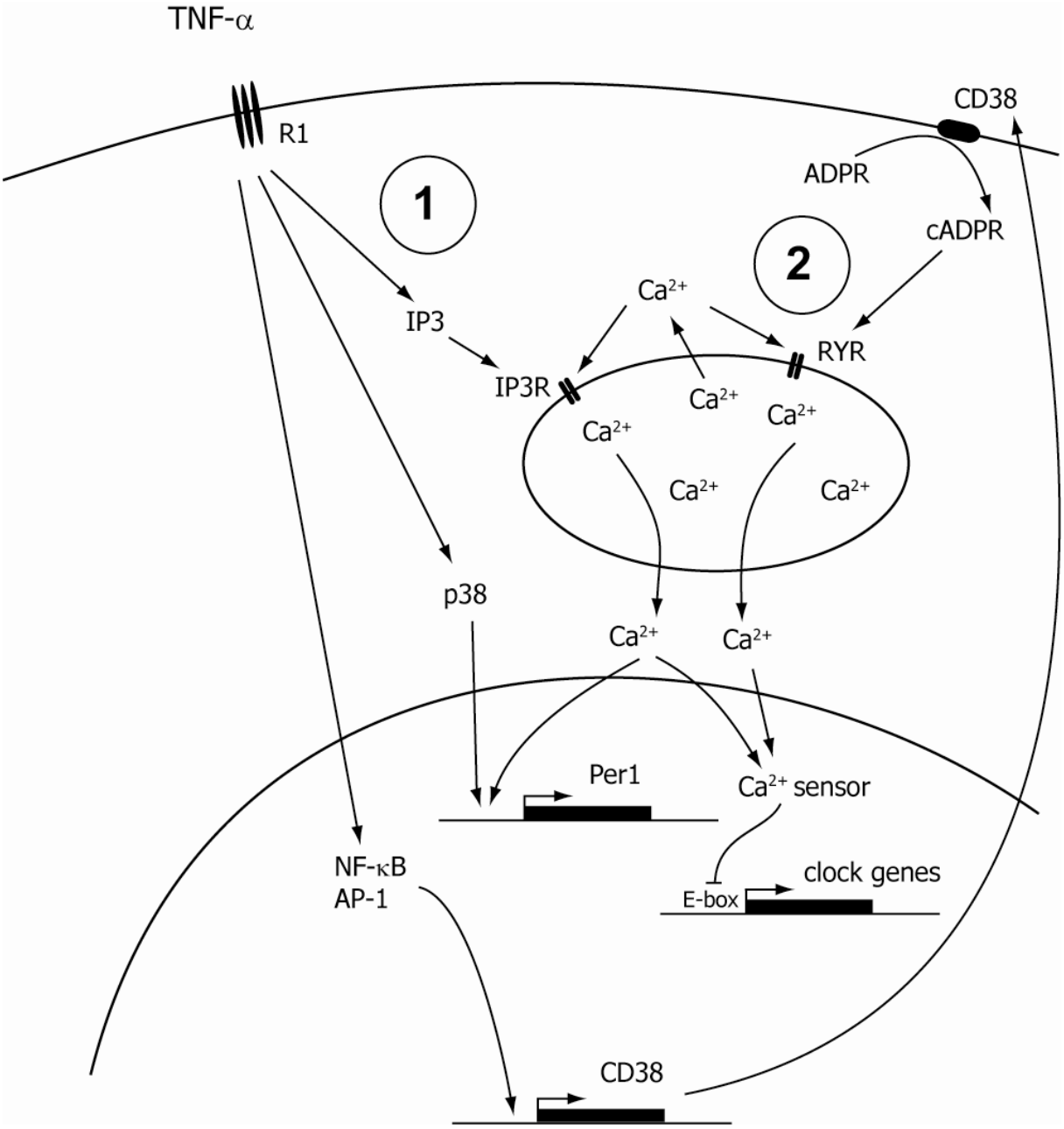

Fig.2: TNF-α signaling leads to alterations in clock gene expression: a possible model

As the central and peripheral clock mechanisms are very similar, if not identical, it is not surprising that the Ca^{2+} signaling also plays a central role in clock gene regulation in the periphery. Although extracellular Ca^{2+} influx may not be as relevant in the periphery as in the SCN neurons, intracellular Ca^{2+} release possibly plays a crucial role in the regulation of clock gene expression. We propose that analogue to the SCN, Ca^{2+} could also be considered as a physiological sensor in the periphery, transmitting immunological and metabolic changes to the molecular clock and thereby coordinating the special needs to specific time windows. We can imagine it being very reasonable to down regulate the clock during an infection. In that way, since cell cycle is closely related to the circadian system, immune cell proliferation, which must occur immediately and frequently, is independent of circadian time windows (163). Similarly, the production of immunological important proteins would be time independent as well. Thus, temporary dampening of the clock may contribute to a fast and effective immune response.

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7. ABBREVIATIONS

ASK	apoptosis stimulated kinase
AP	activator protein
bHLH	basic helix-loop-helix
Bmal1	brain and muscle Arnt-like protein 1
βTrCP	β-transducin repeat containing protein
bZip	basic leucine zipper
cADPR	cyclic adenosine diphosphate-ribose
cAMP	cyclic adenosine monophosphate
CCG	clock-controlled gene
ChIP	chromatin immunoprecipitation
CIPC	clock interacting protein circadian
CK	casein kinase
CLC	cardiotrophin-like cytokine
Clock	circadian locomoter output cycles kaput
CRE	cAMP response element
CREB	cAMP response element binding protein
Cry	cryptochrome
Dec	differentiated embryo chondrocytes
Dbp	D-site albumin promoter binding protein
DD	death-domain
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle medium
DREAM	downstream responsive element antagonist modulator
ER	endoplasmatic reticulum
FASP	familial advanced sleep-phase syndrome
FBS	fetal bovine serum
GSK	glycogen synthase kinase
HAT	histone acetyl transferase
HDAC	histone deacetylase
HIPK	homeobox-interacting protein kinase

Hlf	hepatic leukemia factor
IFN	interferon
I-κB	inhibitor of κB
IKK	inhibitor of NF-κB kinase
IL	interleukin
IP3	inositol 1,4,5-triphosphate
JNK	c-Jun N-terminal kinase
LD	light-dark cycle
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LT	lymphotoxin
MAPK	mitogen activated protein kinase
MEKK	extracellular signal-regulated kinase kinase kinase
NADP	nicotinamide adenine dinucleotide phosphate
NEMO	NF-κB essential modulator
NF-κB	nuclear factor kappa B
NPAS2	neuronal PAS domain protein 2
PAR	proline and acidic amino acid-rich
PAS	Per-Arnt-Sim
Pdxk	pyridoxal kinase
Per	period
PK	protein kinase
PK2	prokineticin-2
PLP	pyridoxal phosphate
PMA	phorbol-12-myristate-13-acetate
PP	protein phosphatase
RIP	receptor-interacting protein
ROR	retinoic acid receptor-related orphan receptor
RYR	ryanodine receptor
SAD	seasonal affective disorder
SCF	SKP1-Cullin1-F-box protein
SCN	suprachiasmatic nucleus
SMC	smooth muscle cell

SNP	single nucleotide polymorphism
Sirt	sirtuin
SODD	silencer of death-domain
Sumo	small ubiquitin related modifier
TACE	TNF-α converting enzyme
Tef	thyrotroph embryonic factor
TGF-α	transforming growth factor alpha
TNF-α	tumor necrosis factor alpha
TNFR	TNF receptor
TLR	Toll-like receptor
TPA	12-O-Tetradecanoylphorbol-13-Acetat
TRADD	TNFR associated death domain
TRAF	TNFR-associated factor

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9. CURRICULUM VITAE

Personal data

Surname:	**PETRZILKA**
Name:	**Saskia**
Date of birth:	12.11.1977
Place of birth:	Geneva, Switzerland
Place of origin:	Winterthur ZH
Nationality:	Swiss

Professional data

2004 – 2008 **University hospital / University of Zurich**

PhD thesis in molecular biology in the lab of Prof. Dr. Med. A. Fontana, section of clinical immunology, department of internal medicine, university hospital Zurich

Participant in the international Ph.D. program in neuroscience, Neuroscience Center Zurich (ZNZ)

1999 – 2004 **University of Zurich**

Master of Science in molecular biology and immunology

Diploma thesis in the lab of Prof. Dr. Urs Greber at the Institute of Zoology
Project: Adenovirus Infection: Interaction of the Capsids with Nucleoporins and Viral DNA Import

Study of Molecular Biology with side subject Immunology

1993 – 1998 Matura type C (mathematics) received at the Kantonsschule Zürcher Oberland, Wetzikon, Switzerland

1989 – 1993 College in Grasse, France

Publication

Cavadini, G.*, **Petrzilka, S.***, Kohler, P., Jud, C., Tobler, I., Birchler, T. & Fontana, A. (2007) *Proc Natl Acad Sci U S A* **104,** 12843-8. *Equally first authors.